GENERIC QUALITY ASSURANCE PROJECT PLAN
For Microbial Source Tracking

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Prepared By:
Natalie Landry, NH Department of Environmental Services

for

USEPA Region I
Office of Environmental Measurement and Evaluation
11 Technology Drive, North Chelmsford, MA 01863

Natalie Landry, NHDES Coastal Watershed Supervisor & Field QA Officer

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Signature Date

Sara Sumner, NHDES Coastal Beach Program Coordinator

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Signature Date

Dr. Steve Jones, UNH JEL Microbiology Lab Director & Laboratory QA Officer

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Signature Date

Tamara Bryant, UNH JEL Microbiology Technician

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Signature Date

Vincent Perelli, NHDES Quality Assurance Manager

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Signature Date

Warren Howard, USEPA Region I Project Officer

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Signature Date

Alan Peterson, USEPA Quality Assurance Officer

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Signature Date

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A3 – Distribution List

The distribution list (table 1) shows the individuals who reviewed and approved the Generic Quality Assurance Project Plan for Microbial Source Tracking and those who just received the QAPP for information. The table also provides the relevance of the reviewer to the QAPP and their contact information.

The New Hampshire Department of Environmental Services (DES) Coastal Restoration Program will distribute additional copies of the Generic Quality Assurance Project Plan for Microbial Source Tracking to all necessary individuals or organizations as requested.

Table 1 QAPP distribution list.

QAPP Recipient Name/Title	Job Relative to QAPP	Organization	Telephone Number/email	Qualifications
Review and Approve QAPP.				
Natalie Landry, Coastal Watershed Supervisor	Manages DES MST Projects & Field QA Officer	NH DES Watershed Management Bureau	603-559-1509 nlandry@des.state.nh.us	On file at NHDES
Sara Sumner, Beach Program Coordinator	Manages DES MST Projects for Beach Program Projects	NH DES Watershed Management Bureau	603-271-8803 ssumner@des.state.nh.us	On file at NHDES
Dr. Steve Jones, JEL Microbiology Lab Director	Supervises sample analysis at JEL and Lab QA Officer	UNH Jackson Estuarine Lab	(603) 862-5124 shj@cisunix.unh.edu	On file at UNH
Tamara Bryant, Lab Technician	Conducts JEL analysis & data management	UNH Jackson Estuarine Lab	603-862-5123 tlbryant@cisunix.unh.edu	On file at UNH
Vincent Perelli, NHDES Quality Assurance Manager	QAPP review	NH DES Planning Unit	603-271-8989 vperelli@des.state.nh.us	On file at NHDES
Alan Peterson, USEPA Quality Assurance Officer	QAPP review	USEPA New England	617-918-8322 Peterson.alan@epa.gov	On file at US EPA
Warren Howard, USEPA Region I Project Officer	QAPP review	USEPA New England	617-918-1587 Howard.Warren@epa.gov	On file at US EPA
Receive QAPP.				
Eric Williams, WAS Supervisor	Keeps QAPP on file	NH DES Watershed Management Bureau	603-271-2358 ewilliams@des.state.nh.us	On file at NHDES
Jody Connor, DES Limnology Center Director	Ensures Beach Program staff receive QAPP	NH DES Watershed Management Bureau	603-271-3414 jconnor@des.state.nh.us	On file at NHDES
Andy Chapman, Limnology Center QA Officer	Keeps QAPP on file	NH DES Watershed Management Bureau	603-271-5334 achapman@des.state.nh.us	On file at NHDES

A4 – Project/Task Organization

Natalie Landry, Coastal Watershed Supervisor, works at the DES Pease Field Office in Portsmouth, NH and is responsible for maintaining the official approved Generic QA Project Plan, Sampling and Analysis Plans, and QA Audits; in addition, she manages microbial source tracking (MST) projects for the Coastal Restoration Program. Sara Sumner, DES Coastal Beach Program Coordinator, is responsible for managing MST projects for the Coastal Beach Program. Water and fecal material sample collection is conducted by DES Staff, Natalie Landry and Sara Sumner, or qualified individuals under Coastal Restoration or Beach Program Staff supervision. Samples are delivered for analysis to the University of New Hampshire Jackson Estuarine Laboratory (JEL). Dr. Steve Jones is the Laboratory Project Manager and QA Officer for the Microbiology Laboratory at the JEL. Samples are analyzed for fecal coliform and E. coli, and the E. coli isolates are further subject to ribotyping analysis using the JEL Qualicon RiboPrinter. Refer to Section B2 for additional information on analytical methods.

All bacterial indicator results are entered into a designated project database at JEL by JEL staff. Dr. Steve Jones is responsible for data review to determine if they meet project objectives and performance criteria. All final bacterial indicator results are sent to the DES Pease Field Office where they are entered into DES Environmental Monitoring Database (EMD). Natalie Landry is responsible for data review to determine if data were entered correctly and designated as final in the EMD, and meet field QA criteria.

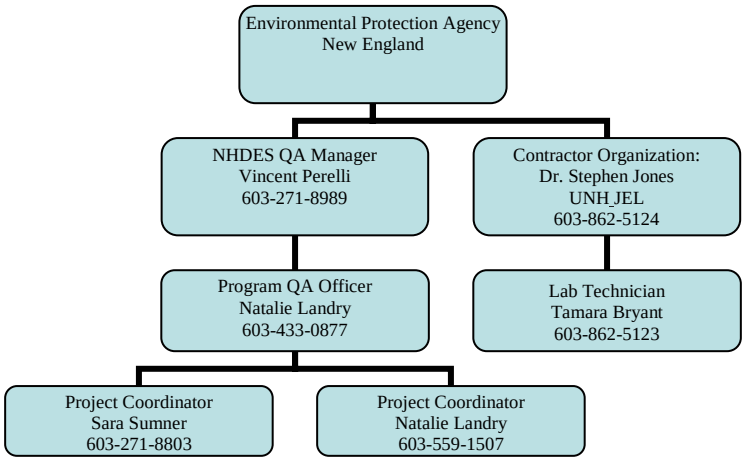
The ribotypes (banding patterns) are generated using the RiboPrinter which is the responsibility of Tamara Bryant. Preliminary ribotyping data analysis is the responsibility of Tamara Bryant; and Dr. Steve Jones conducts the final analysis and interpretation. JEL staff is responsible for a final report that includes the bacterial indicator results, ribotyping results, and laboratory QA information.

DES is the primary user of the collected data. Data are provided to the general public, private sector or other governmental agencies upon request.

Natalie Landry is responsible for QAPP development and revisions. Vincent Perelli is responsible for QA Management at DES. Alan Peterson is the US EPA Quality Assurance Officer. Warren Howard is the US EPA Project Officer.

Natalie Landry is responsible for communicating with the US EPA on technical issues regarding the QAPP. Natalie Landry and Sara Sumner are responsible for communicating project specific objectives, described in project specific Sampling and Analysis Plans, to the appropriate project personnel. Natalie Landry is responsible for communicating problems detected in the data review process to the appropriate parties. Natalie Landry and Sara Sumner are responsible for communicating problems that arise in the field to the appropriate managers and QA personnel. Natalie Landry and Sara Sumner are responsible for communication with Dr. Steve Jones and JEL personnel on sample handling and custody requirements. Dr. Steve Jones is responsible for addressing laboratory analytical problems and communicating those to Natalie Landry or Sara Sumner.

Refer to table 1 for a summary of project participant's roles, organization and qualifications. The organizational chart below illustrates the flow of information between the project participants.



A5 – Problem Definition/Background

Introduction

The DES Watershed Management Bureau (WMB) is responsible for ensuring that the surface waters of the State meet water quality standards. Two programs in the WMB, the Coastal Beach Program and the Coastal Restoration Program, have goals to ensure that the state's surface waters are suitable for recreational uses by evaluating the sanitary quality of surface waters and identifying and eliminating pollution sources that affect the public uses of surface waters, such as swimming, boating, and shellfish harvesting.

DES and UNH, with financial support from EPA, have worked together to develop ribotyping capacity at the UNH Jackson Estuarine Laboratory. Ribotyping is a method of microbial source tracking that provides the actual sources of bacteria, as opposed to just bacteria counts in water samples. This information allows DES to target remediation efforts to the specific contributors of the bacterial pollution that are affecting the polluted waterbody.

Objective

The objectives of DES microbial source tracking projects are to use ribotyping results to: (1) determine the sources of fecal-borne bacterial contamination to surface waters; and (2) determine which sources are significant.

The purpose of this document is to provide assurance that the standard operating procedures for water and fecal sample collection, ribotyping analysis and reporting are of appropriate quality.

Project Setting

The entire geographic area is the State of New Hampshire, and waterbodies in neighboring states that discharge into New Hampshire waters. A subset of the population that we usually develop projects for is coastal watersheds in the 3-50 sq mile range. However, subsets can be catchment areas for small streams, stormwater pipe discharges, and groundwater seeps.

Previous Water Quality Monitoring

The DES Watershed Assistance Section (WAS), which includes the Coastal Restoration Program, has been conducting monitoring in the coastal watershed since the early 1990s. The main focus of this monitoring has been illicit discharge detection surveys and shoreline surveys. Since the late 1990s, the WAS staff have worked with JEL researchers to conduct various pollution source investigation studies.

The DES Coastal Beach Program was initiated in 1996 and includes the monitoring of fifteen coastal beaches. The federal BEACH Act was the impetus for conducting comprehensive monitoring of beaches and initiating pollution sources investigations. The Coastal Beach Program is completing an MST study for several rivers that discharge to popular Atlantic Ocean beaches.

JEL has served the ribotyping needs of northern New England since 2000. JEL has a state-of-the-art laboratory for ribotyping bacterial isolates and has accumulated a source species database for New Hampshire coastal watersheds and various other New England watersheds including Lake Champlain, Vermont, the Palmer River in MA, in Maine's south coastal watershed and Casco Bay, Maine. Dr. Steve Jones has published numerous ribotyping project reports and has presented nationally and internationally on various microbial source tracking projects and issues.

A6 – Project/Task Description

General Overview of Projects

DES is implementing various MST projects to further use this tool to identify the bacteria sources found in contaminated surface water samples. Specifically, isolates of *Escherichia coli* (*E. coli*) are selected from fecal coliform positive samples and analyzed using a microbial source tracking technique (in this case ribotyping). One major goal is to provide DES resource managers with information as to the source animals contributing fecal coliform bacteria contamination to State surface waters. This goal is achieved through the design and

implementation of water sampling, based on prior data to specifically tailor the experimental designs, and ribotyping analysis of bacterial isolates for specific watersheds.

Environmental samples of watershed-specific fecal material from targeted mammalian species are collected to develop local reference libraries. Ribotyping is then used to track sources of microbial contamination in selected water samples from project watersheds. In this process, isolated DNA is extracted and cut by restriction enzymes, separated by gel electrophoresis and then probed for detection of highly conserved rRNA genes. The resulting 'fingerprint' for each unknown isolate is compared to library (database) fingerprints to determine likeness and for identifying sources of fecal contaminants in surface waters.

An anticipated outcome of the MST projects is that DES and local decision makers in the project watersheds have the research-based information that they require to guide action planning for contaminant reduction. Reduction and/or elimination of contamination sources in project watersheds is likely to lower fecal coliform bacteria counts in State surface water used by swimmers and boaters, and for recreational shellfish harvesting.

Project Planning

Project managers consider the use of ribotyping when traditional investigatory methods for bacterial source identification do not provide specific enough information to identify pollution sources. Often data collected by the Watershed Assistance Section or the Shellfish Program are the basis for initiating a discussion about possible MST projects. Meetings are held to discuss the study objectives and to determine if ribotyping is the best method for moving forward with an investigation. If all involved are in agreement with the decision to use this tool for a specific project, a sampling design is discussed as well as other project details required for the Sampling and Analysis Plan (SAP). A planning team typically consists of a project manager from the Watershed Management Bureau as the lead, researchers from the University of New Hampshire JEL and any other agency or municipal staff that have an interest in the study design and outcomes.

Sampling and Analysis Plan

Each DES MST project has a project-specific SAP that contains the preparation date, sample collection procedures, analysis and interpretation information and a description of the reports produced for the project. Each SAP is developed by the project team and submitted to the DES Quality Assurance manager for review and approval. A title and approval page, similar to those required for quality assurance project plans accompanies each SAP. The guidelines for project-specific SAPs are in Appendix J.

Project Timetable

Each DES MST project has a project-specific timetable that is provided in the project-specific Sampling and Analysis Plan (SAP). Each SAP timetable has the anticipated dates of project initiation and completion, expected products, and responsible parties. The timetables include, but are not limited to the following elements: SAP Preparation Date, Sample Collection, Sample Analysis, Data Analysis and Interpretation, and Reporting. The guidelines for project-specific SAPs are in Appendix J.

A7 – Quality Objectives and Criteria for Measurement Data

Table 2 lists the performance criteria for field collection and enumeration analysis of bacterial indicators in water samples collected for the MST projects. Table 3 lists the performance criteria for the ribotyping analysis. Table 4 summarizes the performance criteria for enumeration and ribotyping analyses.

Table 2 Measurement performance criteria for bacterial indicators.

Data Quality Indicators	Measurement Performance Criteria	QC Sample and/or Activity Used to Assess Measurement Performance
Precision-Field	RPD≤ 50%	Field duplicates
Precision-Lab	R< precision criterion (see text below)	Lab duplicates
Representativeness	Project Specific	See Appendix J
Detection limits	1 cfu/100 ml	Sterility tests
Accuracy/Bias	Positive results with positive controls Negative results with negative controls	Positive and negative controls
Comparability	Deviation from SOPs should not influence more than 5% of the data	Data comparability check
Sensitivity	Not expected to be an issue for these projects	N/A
Data Completeness	75% samples collected (on a project basis)	Data completeness check

Table 3 Measurement performance criteria for ribotyping.

Data Quality Indicators	Measurement Performance Criteria	QC Sample and/or Activity Used to Assess Measurement Performance
Precision	Identical patterns at standard settings	Lab strain duplicates
Detection limits	Molecular weights standards	Gel variability control
Accuracy/Bias	Positive results with positive controls Negative results with negative controls	positive and negative controls
Comparability	Deviation from SOPs should not influence more than 5% of the data	Data comparability check
Sensitivity	Optimization and band tolerance settings	Lab strain controls
Data Completeness	75% of samples collected (on a project basis)	Data completeness check

Table 4 Measurement performance criteria.

Parameter	Meas. Range	Precision	Accuracy	Reporting Limit
Bacterial Indicators	>= 1cfu/100mls-10	1 cfu/100mls	1 cfu	1 cfu
Ribotypes	NA	100% similarity for controls	NA	NA

Precision & Accuracy/Bias

The method detection limits, precisions and accuracy for collected data are given in tables 2, 3, and 4.

Field precisions yield RPDs ≤ 50%. If the RPD routinely exceeds 50%, acceptable level may need to be adjusted. This is noted in each final report. Relative percent difference (RPD) is calculated:

$$RPD = \frac{|x_1 - x_2|}{\frac{x_1 + x_2}{2}} \times 100\%$$

where x_1 is the original sample concentration

x_2 is the duplicate sample concentration

Laboratory precision for bacterial indicator measurements is typically determined according to Standard Methods 9020 B-8. (APHA, 1998). The range I for duplicate samples is calculated and compared to predetermined precision criteria. The precision criterion is calculated from the range of log-transformed results for 15 duplicates according to the following formula:

3.27 × (mean of log ranges for 15 duplicates) = precision criterion

The precision criterion is updated periodically using the first 15 duplicate samples analyzed in a month by the same analyst. If the range of ensuing pairs of duplicate samples is greater than the precision criterion, then the increase in imprecision is evaluated to determine if it is acceptable. If not, analytical results obtained since the previous precision check is evaluated and potentially discarded. The cause of the imprecision is identified and resolved.

For ribotyping, precision will be determined by comparing resulting banding patterns for duplicate analyses of ATCC *E. coli* type strain #51739. The patterns should be identical (100% similarity) based on preset optimization and band tolerance settings.

Molecular weight standards are included with each sample batch to allow for comparisons with database patterns. Each sample lane is adjoined by a standard. The RiboPrinter software adjusts, or normalizes banding patterns for samples based on comparisons with the multiple standards included in each sample batch across the whole membrane.

Representativeness

The MST water monitoring for each project identifies and quantifies *E. coli* contamination at numerous locations within the project watershed. Sample sites are carefully selected on the basis of previous water quality data indicating the potential for pollution sources at the site. As such, the resulting data should provide representation of the target sampling areas. Representativeness is also ensured by processing the samples in accordance with the following the established methods, and by obtaining EPA approval of this document.

Comparability

The results for every ribotyping project will be compared to results from previous ribotyping projects. In particular, the degree to which water sample isolates are identified under a given set of ribotyping conditions is a useful comparability guide.

Comparability between samples is achieved through maintaining consistency with SAPs, SOPs, sampling locations, sampling holding times, and sampling methods.

Molecular weight standards are included with each sample batch to allow for comparisons with database patterns. Each sample lane is adjoined by a standard. The RiboPrinter software adjusts, or normalizes banding patterns for samples based on comparisons with the multiple standards included in each sample batch across the whole membrane.

Completeness

Data are collected under a broad range of conditions such as seasonal, weather, and specific tidal stage, providing for a complete data set as set out in the project-specific Sampling and Analysis Plan (SAP). At least five samples are collected for each site or as directed in each project-specific SAP.

At least ten isolates, if ten are present, are selected from each "selected for ribotyping" water sample after enumeration. Banding patterns for each isolate is then determined using the RiboPrinter. Samples with bacterial concentrations that exceed 406 *E. coli*/100 ml is considered a sample "selected for ribotyping." If, due to project constraints, the number of isolates selected for ribotyping is less than 10 per sample, then the project-specific SAP should list the minimum number of isolates and describe the justification for using less than ten isolates per sample.

Although the MST water monitoring projects use a scientific approach to water testing, a statistical sample design has not been implemented. Therefore, all data are not collected for each project. However, a greater number of samples returned to the laboratory provide more data, and consequently more information about the quality of water in the project watershed. Although the MST projects' managers strive to collect and analyze samples from all stations during all "conditions of concern," sampling event completeness is largely determined by weather conditions that affect access to particular sites. Staff is not permitted to collect samples when it is unsafe to do so, for example during periods of extremely high water. Completeness is also determined by the occurrence of "conditions of concern." For example, if three rain events with a minimum rainfall of 0.5 inches within a 24-hour period are the conditions under which the samples must be collected, then completeness may be affected by the occurrence of storms that fit the criteria within the project timetable. At least 75% of the samples set out in the project-specific SAP are collected or as otherwise noted in the SAP.

The laboratory will accept an *E. coli* confirmation level of >75%. At least 90% of confirmed *E. coli* isolates should yield detectable banding patterns. Of these successful ribotyped isolates, at least 85% will be accepted as *E. coli*, determined by RiboPrinter analysis.

Sensitivity

For indicator organisms, we are interested in whatever range we can detect. However, at the low end, concentrations that are below the detection limit of 1 cfu/100 ml are not of interest because of the low level of loading to estuarine waters that such numbers represent. To increase the sensitivity of bacterial analysis, filtration of larger volumes is required. However, volumes >100 ml typically cause clogging problems with membrane filters and is avoided if at all possible.

For ribotyping isolates, JEL decides the acceptance of a source species match to a water sample isolate by choosing the source species profile with the best similarity coefficient (Dice's coincidence index) at a given set of optimization and tolerance settings. The most recent UNH published report for New Hampshire had the optimization set at 1.56% and band position

tolerance set at 1.00%. These two parameters were used to adjust the ability to differentiate between bands for the degree of accuracy desired, and also to compensate for possible misalignment of homologous bands caused by technical problems. The DES MST projects will use an optimization set at 1.5% and band tolerance set at 1.00%.

Quantitation Limits

The analytical method, analytical/achievable method detection limit, and the analytical/achievable laboratory quantitation limits for this project are shown in table 7.

Because *E. coli* cultures for use in the RiboPrinter are prepared in a consistent fashion and the RiboPrinter will not run samples with inadequate amounts of DNA, rRNA genes will always be present in detectable quantities for ribotyping analysis. The RiboPrinter produces data for different molecular weight pieces of DNA (molecular weight standards) based on detection of the probe by the camera, with the densiometry data translated into digital format. The densiometry data are transformed into DNA bands using GelComaprII software, based on areas in the image that are most dense and above background threshold densiometry.

A8 – Special Training/Certification

Water sample collection occurs according to the sampling methods outlined in section B2 (Also see Appendix A for the Standard Operating Procedure for Bacterial Indicators Sample Collection). The Coastal Watershed Supervisor (CWS) and Coastal Beach Program Manager (CBPM) are responsible for training any personnel in water sample collection according to this document. Training of personnel in water sample collection takes place prior to the initial sample collection date for each project. In addition, CWS or CBPM accompanies any personnel involved to directly oversee and assure proper water sample collection. New personnel are trained prior to water sampling. A list of people trained in water sample collection is kept on file at the DES Pease Field Office.

Fecal material sample collection occurs according to the sampling methods outlined in section B2 (Also see Appendix B for the Fecal Sample Collection SOP). The CWS and CBPM are responsible for training any personnel in fecal material (scat) collection according to this document. Human, domestic animals and livestock scat samples are collected immediately following defecation to ensure accurate identification. Accurate identification of wildlife and avian source species may require more formal staff training.

The bacterial analyses for these projects is conducted at the JEL Microbiology Lab. The analyses is an *E. coli* membrane filtration method. The SOPs for the bacterial indicators are included in Appendix C. Dr. Steve Jones is responsible for training and monitoring laboratory staff and taking all corrective actions, as necessary.

Ribotyping analysis and interpretation occur according to the methods outlined in section B2 (Also see Appendix D for the Ribotyping Analysis and Interpretation SOP). Only Dr. Steve Jones and Tamara Bryant are trained to both analyze and interpret ribotyping data. New staff is

thoroughly trained prior to conducted ribotyping analysis and interpretation for DES MST projects.

A9 – Documents and Records

The final version of the Generic Quality Assurance Project Plan for Microbial Source Tracking is stored electronically in the Watershed Management Bureau data management system. A hard copy is available at the DES Pease Field Office in Portsmouth, NH. The Coastal Restoration Supervisor solicits comment before any changes are made to the document. All changes are submitted to the DES QA Manager and EPA for approval. All projects operating under the Generic QAPP require an approved specific site plan, referred to as Sampling and Analysis Plans (SAPs) hereafter. The guidelines for SAPs are contained in Appendix J. An annual review of the QAPP will occur each year and a full revision will take place every five years.

Office

A station ID form is completed for each station using the DES Sampling Station Identification Form (Appendix E). The data from the form is entered into the DES Environmental Monitoring Database and kept on file by the project manager, either the Coastal Watershed Supervisor or the Coastal Beach Program Manager.

Field

A Water Sample Collection Field Sheet (Appendix F) is completed during each water sample collection event. A Fecal Sample Collection Field Sheet (Appendix G) is completed during each fecal sample collection. The appropriate information is transferred to the Water & Fecal Material Monitoring QA Sheet and Delivery Form (Appendix H). The field sheets are kept on file by the CWS. If the CBPM is the project lead on a particular MST project, copies of the field sheets are provided to the CWS for QA Audits. If water and fecal samples are collected during the same field work, a separate Water & Fecal Material Monitoring QA Sheet and Delivery Form is completed for each matrix.

Laboratory

DES MST projects use the field sheets mentioned previously when collecting water or fecal material samples. When samples are delivered to JEL, information such as the chain of custody, acceptance criteria, and sample matrix are recorded on the Water & Fecal Material Monitoring QA Sheet and Delivery Form. JEL retains the original Form and returns a copy of the original with sample results to the DES CWS. The original Sample Collection Field Sheets are kept by DES and a copy is given to the laboratory with the samples, in addition to the QA Field Sheet and Delivery Form. Copies of the original records are archived by the DES CWS at the end of each project.

The Microbiology Laboratory Quality Assurance Plan for JEL (Appendix I) describes the record keeping procedures for the laboratory. Bound laboratory notebooks are used for entering sample information into the laboratory records. Information is filled out in ink, dated, and the person entering the information includes their name on the pages. The notebooks are stored in the analytical laboratory and records throughout the holding time of the samples are maintained in them. After each batch of samples is analyzed, the results are recorded into spreadsheet databases. After the bacterial analyses are completed, JEL will complete the Water & Fecal Material Monitoring QA Field Data Sheet and Delivery Form and send a copy to the CWS along with the analytical results.

Ribotyping data are generated by the RiboPrinter. All data are analyzed with GelComparII software on a Dell computer, where the source species database is also stored. Hard copies of ribotype patterns and similarity coefficients for the unknown and its most closely related source species are printed for interpretation. Interpretation and accompanying graphical representations of the data are done using MS Excel on Macintosh computers. The data and graphics are stored in a project-specific file in MS Excel on Macintosh computers. Results (tabular, graphic and interpretive text) are delivered to the CWS or the CBPM in a bound report and an electronic file in either MS Word or pfd format.

B1 – Sampling Process Design

Water samples are collected to monitor the quality of surface waters and discharges on a pre-scheduled basis, as well as following rainfall events or other conditions of concern as directed in the project-specific SAP. Typically, sampling site locations are pre-determined by previous studies or monitoring efforts. Sampling sites are selected to isolate potential sources of contaminants or to characterize the sources in a waterbody. Each SAP will provide descriptions and location information for the sampling sites. In addition, each SAP will list the animals in the watershed targeted for fecal material sample collection. Instructions for creating a SAP are located in Appendix J.

All water and fecal material samples are analyzed for *E. coli* according to Standard Procedure for Detection of Total Coliforms, Fecal coliforms, Escherichia coli and Enterococci from Environmental Samples (Appendix C).

B2-Sampling Method

This section describes the various sampling methods for water, fecal material and air. Table 5 which is located at the end of the section describes the sampling requirements for each parameter measured in MST projects.

Bacteria Sample Collection

The SOP for water sample collection (Appendix A) describes the appropriate sampling procedure for collecting water samples for bacteriological analysis. Collection procedure will

adhere to this SOP with the exception of using sterile 18-oz. Whirl-Paks® instead of 250-mL HDPE bottles. Individual project Sampling and Analysis Plans may refer to other water sample collection SOPs as appropriate. If SOPs other than those currently present in the Generic QAPP for MST are used, a copy of the relevant SOP is attached to the project-specific SAP for documentation purposes.

Whenever possible, the collector samples so that the water body is not disturbed from wading. All samples are taken as close to approximate mid-stream as possible. If it is necessary to wade into the water, the sample collector stands downstream of the sample, taking a sample upstream. If the collector disturbs sediment when wading, the collector waits until the effect of disturbance is no longer present before taking the sample. Samples are collected in 18-oz. Whirl-Paks®. On the Whirl-Paks®, the sample date, sample site identification and collector's name are recorded using water proof/ indelible ink. Samples are stored in a cooler, on ice-packs and transported to the lab within 6 hours for analysis within 8 hours of sample collection.

The sample water temperature is measured and recorded when samples are delivered to the laboratory to confirm that the proper temperature was maintained, preferably between 0-10°C, during sample collection and transport. Water samples delivered to the laboratory at a temperature greater than 10°C are not rejected. For example, samples collected from ambient waters measuring 25°C and immediately delivered to the laboratory are likely not to have cooled to less than 10°C during transportation. Such samples are deemed acceptable, especially if the temperature at time of delivery is less than that at the time of collection. The decision to accept or reject samples with a temperature of over 10°C at the time of delivery is exercised with caution and at the discretion of JEL and DES.

Field Water and Air Analysis

Sample collection date, weather, and air temperature is recorded on the Water Sample Collection Field Sheet (Appendix F). Air temperature is measured using a Reotemp, stainless steel, bi-metal thermometer or equivalent. At each sampling site, collection time, water temperature, and other observations that may have an impact on water quality are recorded on the field sheet.

Water temperature at each sample site is measured using a Reotemp, stainless steel, bi-metal thermometer or equivalent. Water temperature is measured by placing the thermometer in the water until the thermometer reading has stabilized. The temperature is measured by looking squarely at the face of the thermometer. The thermometer is calibrated annually at a minimum.

Fecal Material Collection

The SOP for fecal material sample collection (Appendix B) describes the appropriate sampling procedure for collecting fecal material samples for bacteriological analysis. The fecal material sample is collected using 18-oz. Whirl-Paks®. Careful species identification is made when collecting wildlife or avian scat. Reference guides are listed in the SOP.

Laboratory Analysis

JEL analyzes the water and fecal material samples for *E. coli* concentrations and ribotyping.

Table 5 Sample Requirements.

Parameter	Collection Method	Matrix	Sample Container	Sample Preservation	Holding Time	Method Reference
E. coli	Grab	Water	sterilized Whirl-Pak®	Cool, 0-10°C	6 hours	Appendix A
E. coli	Grab	Fecal material	sterilized Whirl-Pak®	Cool, 0-10°C	6 hours	Appendix B
Water Temperature	Measured In-situ	Water	If necessary, sterilized Whirl-Pak®	N/A	N/A	QAPP, Section B2
Air Temperature	Measured In-situ	Air	N/A	N/A	N/A	QAPP, Section B2
Ribotyping	Isolates from a plate	Tryptic soy agar	RiboPrinter sample carrier	-80°C	indefinite	Appendix D

B3- Handling and Custody Requirements

Bacteria sample collection is performed by the appropriate trained personnel. Samples are transferred to a cooler with ice packs to initiate the preservation process. Samples are transported by vehicle to JEL within the proper holding times (Refer to table 5). The appropriate field data sheet information is transferred to the Water & Fecal Material Monitoring QA Sheet & Delivery Form. Chain of custody is recorded on the field sheets and the QA Sheet and Delivery Form (Appendices F, G, and H). This is completed when samples exchange hands between sample collection and delivery to the laboratory. Water temperature of the samples is measured at the time of sample delivery. A copy of the original field data sheet and the Water & Fecal Material Monitoring QA Sheet & Delivery Form is given to the laboratory with the samples. Following completion of the water sample analysis by JEL, laboratory results are forwarded to the DES CWS.

B4-Analytical Methods

The bacterial analyses for DES MST projects is conducted at the JEL Microbiology Lab and is in accordance with an *Escherichia coli* membrane filtration method. The SOPs for the bacterial indicators are included in Appendix C. The reference limits for each bacterial indicator are listed in table 7. Ribotyping analysis is also conducted at JEL and the reference limits are provides in table 7, along with the ribotyping SOPs in Appendix D. Dr. Steve Jones will be responsible for all corrective actions and will also be responsible for all non-standard method validation.

Table 6 Water bacterial indicators and ribotyping reference limits.

Indicator	Analytical method SOP Reference	Project Action Level	Analytical/Achievable Method Detection Limit	Project Quantitation Limit
Escherichia coli	Membrane Filter Procedure, EPA 600/4-85/076; Standard Method 9213D.3 (APHA, 1995) A-1	406 cts/100ml	0+ cts/100 mL (depends on dilution and sample volume)	0+ cts/100 mL (depends on dilution and sample volume)
Ribotyping	Refer to Appendix D	NA	NA	NA

B5-Quality Control Requirements

All biological monitoring analyses performed adhere to the quality control (QC) guidelines listed in the Standard Operating Procedures (Appendices A, C, D and I). Water and air temperature data are exempt from QC measures. Table 8 summarizes laboratory and field quality control samples. Field duplicates are collected at a rate of at least 10% of the total number of water samples collected during a single day of collection.

Table 7 Field and laboratory quality control samples.

Analyses	Field QC	Laboratory QC	Data Quality Indicators	Acceptance Limit Field	Acceptance Limit Lab	Corrective Action
E. coli	Field duplicates	Laboratory duplicates	Precision	RPD<50%	Appendix C	Appendix C
Water Temp.	None	N/A	N/A	Total precision not measured	N/A	QAPP, Section B2
Air Temp.	None	N/A	N/A	Total precision not measured	N/A	QAPP, Section B2
Ribotyping	N/A	NA	Per batch	N/A	100% similarity	Appendix D

When bacterial indicator laboratory results are acceptable, the laboratory acknowledges this by signing the Water & Fecal Material Monitoring QA Sheet and Delivery Form. JEL staff signs the line labeled “Report Verifier” under lab QA section (Appendix H).

B6/B7 – Instrument/Equipment Inspection, Maintenance and Calibration

The field instrument used during water sample collection is a Reotemp thermometer. The Reotemp thermometer is calibrated annually at a minimum. Refer to Appendix K for the Standard Operating Procedures for thermometer calibration. The date of calibration is recorded on a piece of tape attached to the thermometer.

Laboratory instruments and equipment are inspected, maintained and calibrated by the laboratory. Refer to the UNH JEL Microbiology Laboratory Quality Assurance Plan (Appendix I) for additional information on laboratory instruments and equipment. All documents are on file at the laboratory in Durham. Table 9 summarizes inspection, maintenance and calibration requirements.

Table 8 Laboratory instruments.

Equipment name	Activity	Frequency of activity	Acceptance criteria	Corrective action	Person responsible
Lab Line EnviroShaker Model 3597 LB incubator (35°C)	Check temperature	Daily before use and at end of day	Meets analytical requirements	Adjust temperature control and confirm stability of acceptable temperature	JEL-Micro
VWR Model 1510 E incubator (41°C)	Check temperature	Daily before use and at end of day	Meets analytical requirements	Adjust temperature control and confirm stability of acceptable temperature	JEL-Micro
Fisher Isolatemp Incubator (44.5°C)	Check temperature	Daily before use and at end of day	Meets analytical requirements	Adjust temperature control and confirm stability of acceptable temperature	JEL-Micro
Seaward Stomacher 80 Lab System	Clean instrument	Before and after every use	Meets analytical requirements	Re-swab with alcohol	JEL-Micro
RiboPrinter	Clean instrument	Prior to each project/daily/3 months	Automated acceptance of conditions	Repeat; reference online technical support	JEL-Micro
RiboPrinter	Maintenance as part of manufacturer contract	twice yearly	Determined by manufacturer	Made by manufacturer	S. Jones

B8-Inspection/Acceptance Requirements for Supplies and Consumables

Laboratory and field personnel inspect the Whirl-Paks® before sample collection. Possibly contaminated Whirl-Paks® are discarded.

B9-Non-direct Measurements

Tidal data are used in making decisions on when to sample monitoring sites that are accessed by boat or that are sampled at a specific tidal stage. For monitoring sites accessed by boat, water samples are collected only when it is possible to get a boat to the site without being stranded by low water. Data on time of low tide are acquired from National Oceanic and Atmospheric Administration tide charts, using times for the Portland, ME base station. Using this information and the tidal lag for each sampling site, the appropriate tidal conditions for sampling are determined.

Rainfall data are used in reports to determine total daily precipitation, when conditions of concern include rain events. Primary weather stations in the coast from which data are acquired are located in Portsmouth (Pease International Airport), and Seabrook (North Atlantic Energy Service Corporation), NH. These data are kept on file in MS Excel Spreadsheets by the DES Shellfish Program and are available to DES and JEL staff upon request. Data from stations in Durham (University of New Hampshire) and Greenland (National Weather Service) are used as needed.

B10-Data Management

For each sampling event, record keeping and information flow originates from the DES Project Manager (as assigned in the SAP), as some elements of the QA Sheet & Delivery Form (e.g., Project Name, watershed, Portland tidal data) and the Field Collection Sheets (e.g., Project Name) are recorded prior to the sampling event. Information collected by field personnel is added to the sheets during the sampling event. The original QA Sheet & Delivery Form and a copy of the Field Sample Collection Sheets are then given JEL personnel along with water samples. A completed copy of the QA Sheet & Delivery Form is returned to the DES upon completion of laboratory results. The Form is then used by DES for data entry and QA procedures. All hard copies are kept on file by the Project Manager in a file drawer labeled "MST Projects."

Natalie Landry is responsible for data entry. All bacterial indicator concentration data are managed in DES EMD. As data are entered, the appropriate section of the QA Sheet & Delivery is initialed and dated. As data entry is verified, the entry in the database field entitled "Status" is changed from a "Preliminary" (the default value) to "Reviewed," and the appropriate section of the QA Sheet is initialed and dated. The "Status" field is changed from "Reviewed" to "Final" after the project QA report is completed.

The QA Sheet & Delivery Form is located in Appendix H and the two field collection sheets for water and fecal material samples are located in Appendix F and G, respectively. Data are retrieved for, and distributed to, interested parties upon request.

C1-Assessments and Response Actions

The Project Manager (Natalie Landry or Sara Sumner) will monitor all aspects of the field program. Although there are no planned internal audits or assessments, if at any point

water sampling procedures or results come under question, problems are assessed and immediately remedied by DES Staff.

All field sampling procedures are observed by Natalie Landry and/or Sara Sumner during each sampling event. Any observed deviations from the sampling procedures prescribed in the QAPP are corrected at the time of observation.

All laboratory QA data and results are included in each project-specific report produced by JEL. All JEL records are maintained for a minimum of three years and are archived electronically and as hard copies in the project manager's office. Any deficiencies will be assessed and remedied by JEL Staff.

C2-Reports to Management

Annual QA Audit reports are completed and sent to Vincent Perelli, NHDES Quality Assurance Manager. Other reports are listed in the project-specific SAP documents. All reports are kept on file and made available for review at the DES Pease Office.

D1-Data Review, Verification and Validation

Data Representativeness: The program manager or CWS reviews all data generated. Data not meeting stated RPDs may be rejected upon consultation between JEL and DES.

Documentation, Data Reduction and Reporting: Field data sheets and QA Sheet & Delivery Forms are reviewed to determine adherence to chain-of-custody and sample holding times. Chain-of-custody documentation is maintained at the DES Pease office.

Corrective Action: When it is found that the data are incomplete or that the results are unacceptable, DES staff may determine that one or more of the following procedures for corrective action is undertaken.

1. Incomplete data – Omissions on laboratory data reports may place the entire analysis in question. Incomplete lab data may call for re-introduction or re-analysis of the questionable sample, if possible. If not possible, invalid entries are not incorporated into the useable data set.
2. Conflicting data – Conflicting data may put the entire analytical performance in question. A site investigation may be required to determine if other variables are influencing the results. DES staff may require re-sampling or re-analysis, depending on the circumstance.
3. Poor performance – When results appear to be abnormal, DES staff reviews the available data and discusses the problem with the lab. Upon examination, all or some of the following actions may be applied:
   A. DES staff will discuss laboratory procedures with JEL QA Officer and staff.
   B. DES staff will examine water sampling technique.

D2-Verification and Validation Procedures

Initial data review is performed by Natalie Landry upon receipt of completed QA Sheet and Delivery Form and data report from the laboratory. This initial review is designed to detect obvious errors primarily through identification of unrealistic or unusual entries on the data sheet according to the steps listed in D1. Following this review, data are entered in the DES Environmental Monitoring Database, and each record is flagged as preliminary. Following data entry, the Project Manager or other DES staff assists Natalie Landry in data entry verification/ validation by reading over all entries on the data report. Data that are deemed invalid by the DES Project Manager because of errors discovered through the initial review, or because of comments from the analytical laboratory, are retained in the database with appropriate explanatory comment and are marked as "Invalid".

All samples used in ribotyping analysis are cross-checked between the RiboPrinter technician and the data analysis technician to ensure completeness. The results for each statistical analysis of each water and fecal sample isolate are printed in hard copy and pertinent data recorded in a spreadsheet. The project manager reviews the summary spreadsheet for accuracy by comparison to hard copy analytical results.

D3- Reconciliation with User Requirements

Data are generated based on the quality objectives outlined in Section A7, Tables 2 and 3 and verified according to sections D1 and D2. Limitations in the data are clearly defined for potential end users in all reports produced.

Appendix A Standard Operating Procedure for Bacterial Indicators Sample Collection

Prepared by Natalie Landry

Based on the following publications:

Public Heath Significance of Stormwater-Borne Microogranisms by Stephen Jones, University of New Hampshire. April 1999.

Quality Assurance Project Plan For Shellfish Sanitary Surveys by Andrew Chapman and Chris Nash, NHDES. July 2002.

NHDES Beach Program Generic Quality Assurance Project Plan by Sara Sumner, NHDES. May 15, 2002.

Background Information and Uses:

Bacterial Indicators are certain bacterial species used to indicate the presence of fecal contamination in surface waters. An *ideal* fecal indicator should occur only in the presence of fecal contamination, not be capable of growth in the environment, be present consistently and in high concentrations in fecal material, have concentrations that are statistically related to concentrations of pathogens, be present in greater numbers than pathogens in the environment, have greater survival than pathogens under unfavorable environmental conditions and during disinfection, and the detection method should be easy and inexpensive, and sensitive, accurate, precise and specific. No ideal indicator has yet to be developed. In fact, many of the bacterial indicators are capable of growing in the environment, are sensitive to disinfection and environmental stress, or do not correlate with viruses. Thus, a sampling and analytical approach that includes a suite of indicators that address different issues is probably the best strategy to assess the sanitary conditions of surface water (Jones, 1999). The State of New Hampshire uses three indicator species depending on the type of surface water and the designated use for the waterbody. Testing for bacterial indicators has proven to be an effective indicator of fecal contamination in DES investigations of dry weather discharges, stormwater and during shoreline surveys. Below is a list of pollution sources that bacterial indicators can help identify:

- Faulty septic systems
- Sewage exfiltration
- Illicit discharges into the storm drainage system
- Agricultural runoff

A. Equipment

1. Sterile 250-mL HDPE bottles.
2. Protective gloves (large plastic gloves or small laboratory latex gloves) and protective eye wear.
3. If working in roadways, traffic cones, reflector vest, a pick and shovel. Steel-toed boots are recommended.

4. If working in waterways, chest waders or rubber boots are recommended.
5. Knife.
6. Sampling pole.
7. Cooler and ice packs or ice.

B. Sample Collection

In the office:

1) Check weather. Accuweather (http://www.accuweather.com) and NOAA (http://www.erh.noaa.gov/box/dailystns.shtml) are two suggested websites to check past weather data.

   a.) For Dry Weather Sample Collection: Check the weather to confirm that it has not rained more than 0.10 inches in the past 48 hours at the sampling site.

   b.) For Wet Weather Sample Collection: Check the weather to confirm that it has rained at least 0.3 inches of rain within the past 24 hours at the sampling site.

2) Note estimated number of samples and type(s) of indicator species for the day on the DES Laboratory Services Unit (LSU) blackboard.
3) Complete the following actions in the office/LSU: place masking tape/label on the bottles, place ice/ice packs into cooler, assemble bottles, markers, field datasheet/notebook, Sampling Station Identification Form, clipboard, gloves, protective eye wear, safety vests, cones, pick, shovel, sampling pole, knife, and hand sanitizer for field use.

In the field:

Always collect samples from the most downstream sampling location first and work up stream. This applies to storm drainage systems sampling, too.

Sampling from a catch basin, manhole, stormdrain outfall or other pipe

1. If sampling from a catch basin or a manhole, use traffic cones and a reflector vest to protect yourself from moving traffic. Open the catch basin or manhole cover using a pick and shovel; you may need someone to help. Steel-toed boots are recommended.
2. Wear protective gloves (large plastic gloves or small laboratory latex gloves), and if splashing is likely to occur, protective eyewear should also be worn.
3. Use a sterile 250-mL HDPE bottle from the DES lab to collect the sample.
4. Write the sample ID number, the date of collection, time of collection, indicator species, dilution factor and sampler's initials on a label using water proof/indelible ink.
5. Open bottle and make sure cap does not get contaminated as you collect the sample. If possible, place bottle underneath the pipe to capture flowing water. If the pipe is flush with the water surface, place the bottle in the pipe, and try to capture representative water. Try not to disturb sediments in the pipe. If the sediment is disturbed, collect the sample away from the disturbed

area to minimize contamination possibilities. If sample collection away from the disturbed area is not possible, note this on the field data sheet.

6. If sampling location is inaccessible, a sampling pole may be used. Place a bottle in holder and lower bottle to collection site.
7. If the water level is extremely low, the top of the bottle can be cut with a knife, and this bottle can be used to scoop water. Collected water should be dumped into a new bottle with a cap.
8. The DES lab needs at least 100 mL of water to be able to analyze the sample. The bottle may need to be shaken to remove water, allowing for a one-inch air space.
9. Cap the bottle.
10. Place bottle in a cooler on ice as soon as possible.
11. Wash hands thoroughly following sampling.
12. Bring sample(s) to the lab before 3:00 PM.

Sampling from a stream or river

1. Wear protective gloves (large plastic gloves or small laboratory latex gloves), and if splashing is likely to occur, protective eyewear should also be worn.
2. Use a sterile 250-mL HDPE bottle from the DES lab to collect the sample.
3. Write the sample ID number, the date of collection, time of collection, indicator species, dilution factor and sampler's initials on a label using water proof/indelible ink.
4. Place a bottle onto a sampling pole. Open bottle and make sure the cap does not get contaminated as you collect the sample. Be careful not to disturb the bottom sediments. If the sediments are disturbed, collect the sample away from the disturbed area to minimize contamination possibilities. If it is not possible to collect the sample collection away from the disturbed area, note this on the field data sheet.
5. With a downward motion and then upstream thrust, dip the bottle to mid-depth (at least a foot below the surface) and mid-channel in the stream. Fill the bottle with a sweeping motion, continuing upstream. In streams or rivers in which it is difficult to collect a sample at the desired depth, locate the deepest area with a moving current. Always collect sample moving against the current to reduce the chance of contamination.
6. The DES lab needs at least 100 mL of water to be able to analyze the sample. The bottle may need to be shaken to remove water, allowing for a one-inch air space.
7. Cap the bottle.
8. Place bottle in a cooler on ice as soon as possible.
9. Wash hands thoroughly following sampling.
10. Bring sample(s) to the lab before 3:00 PM.

Sampling from a groundwater seep

1. Wear protective gloves (large plastic gloves or small laboratory latex gloves), and if splashing is likely to occur, protective eyewear should also be worn.
2. Use a sterile 250-mL HDPE bottle from the DES lab to collect the sample.
3. Write the sample ID number, the date of collection, time of collection, indicator specie(s), dilution factor and sampler's initials on a label using water proof/ indelible ink.
4. Place a bottle onto a sampling pole. Using a pole for collecting samples from seeps is not always necessary. Open bottle and make sure the cap does not get contaminated as you collect the sample. Be careful not to disturb the sediments. If the sediments are disturbed, note this on the field data sheet.

5. Hold the bottle, using the pole, into the flow of the discharging seep. Allow the bottle to fill.
6. The DES lab needs at least 100 mL of water to be able to analyze the sample. The bottle may need to be shaken to remove water, allowing for a one-inch air space.
7. Cap the bottle.
8. Place bottle in a cooler on ice as soon as possible.
9. Wash hands thoroughly following sampling.
10. Bring sample(s) to the lab before 3:00 PM.

Field Data Sheets:

1. Complete the project specific field data sheet immediately before or after collecting each sample. The project specific field data sheet should include, at a minimum, the following information: Date and time of sample collection, town name, direct and downstream waterbody affected, sampler's name or initials, site description information, and observations. Other information could include the geographic reference, past rainfall/weather and other field measurements.
2. If the sampling location is a new site, complete a Sampling Station Identification Form in addition to the field data sheet.

Field Duplicates:

1. If you collect greater than five to nine samples during the day, collect one duplicate sample. If you collect ten samples or more during the day, collect duplicates at a rate of 10%.

Back at the LSU:

1. LSU staff will measure the temperature of one of the samples to ensure samples were kept cool enough during delivery.
2. Transfer the sample information from the field data sheet onto the NH DES Laboratory Services Login and Custody Sheet.
3. Place the samples in order according to the time samples were collected on the bench in the LSU log-in room.
4. Place the appropriate labels on the bottle caps. These labels inform lab personnel of analyses to be run.
5. Sample dilution is required. Dilutions are X1, X10, X100, or X1000. Indicate the dilution factor by listing it on the label. Login sheets must also be labeled with the dilution factor(s) in the other/notes section. Dilution levels are chosen by estimating the bacteria levels in the sample and referring to the table below. If there is no prior knowledge of the site, the default dilution level is X10.

Dilution level	**Minimum detection limit (cts/100 mL)**	**Maximum detection limit (cts/100 mL)**
× 1	0	> 200
× 10	< 10	> 2,000
× 100	< 100	> 20,000
× 1,000	< 1,000	> 200,000

6. Sign the custody sheet to relinquish the samples to the laboratory. The lab personnel must review and sign the custody sheet. Always notify lab personnel when you drop samples off.

D. Analysis

The laboratory will analyze the water samples for the selected bacterial indicator(s) which include *E. coli*, enterococci and fecal coliform. Following completion of water sample analysis, laboratory results are forwarded to the DES Watershed Assistance Section for entry into the water quality database.

E. Data Interpretation

The bacterial indicator results should be reviewed by the project manager. The following table and text provides a guide for the manager when deciding future steps. Professional judgment and consultation with other staff, in addition to or in replacement of the table and text below, are acceptable methods for arriving at a decision.

Table 1: Suggested Guidance for Action

Bacterial Indicator	**Selection of indicator based on waterbody sampled**	**Action Level**	**Minor Observations**	**Major Observations**
E. coli	Freshwater	88	High Flow Color Vegetation Deposits/Stains Damage to pipe	Odor Grey mat Floatable matter Turbidity
Fecal Coliform	Tidal waters, including Shellfish growing waters	43		
Enterococci	Tidal waters	104		
Action Levels are based on Surface Water Quality Standards; however, State Water Quality Standards do not apply to discharges from pipes. The only purpose of Action Levels is to provide guidance to investigators.				

1. If the Action Level for the first sample is not exceeded and no Observations are noted, do not collect any more samples. If the Action Level for the first sample is not exceeded and any Observations are noted, collect a second sample. If the Action Level for the first sample is exceeded, collect a second sample.
2. If the Action Level for the second sample is not exceeded and no Observations are noted, do not collect any more samples. If the Action Level for the second sample is not exceeded and only Minor Observations are noted, do not collect any more samples. If the Action Level for the second sample is not exceeded and Major Observations are noted, consider this site for further investigation. If the Action Level for the second sample is exceeded, consider this site for further investigations. If the Action Level for the second sample is exceeded and any Observations are noted, consider this site for further investigation.
3. Further investigations could include dye testing, smoke testing, ribotyping, discussions with property owners and/or town officials, sampling upstream to try to identify the source, sampling and testing for other contaminants, and optical brightener testing.

Appendix B Standard Operating Procedures for Scat Collection

SOP updated on 6/23/2004 by Natalie Landry, NHDES

SOP reviewed on 2/10/2003

SOP developed by Andrew Chapman, NHDES

Strategies for Finding Scat:

There are two general approaches to finding scat. In an area that most likely has large animal or flocking bird populations; a broad sweep of an area may first be conducted. This technique can be quite successful for finding many types of scat when there is a significant diversity of species. In areas with small animals or solitary birds selecting a specific specie and scat is most effective.

Basic elements of animal and/or bird behavior may be predictable in that they need food and water and try to avoid being preyed upon. Standing or flowing water bodies and wetland systems are most often considered excellent water sources. Fruits, nuts, seeds, soft vegetation, invertebrates and vertebrates may be some common food sources. To avoid being seen and/or eaten, animals and birds seek habitats that offer good protection and if possible also serve as a "lookout" for approaching predators. This ideal habitat varies specie to specie. However, edges of wooded tracts of land, banks of streams and rivers, thick grassy or low vegetation, stone walls, stumps and logs, etc. are often productive areas for finding scat. For some species, the before mentioned also provide ideal locations for depositing scat to mark their territory, both for themselves and others.

The presence of animals or birds prior to scat location is often determined by finding signs before locating the scat. Animal and bird sign can be broken down into those seen, heard and smelled. Sign which can be seen includes tracks, runs, feed remains/areas, caches, rubs, hair, fur, feathers, lays, nests and dens. Sign that can be heard includes calls, and movement of body parts such as wing beats, beak pecking or clapping, movements through dry wooded or leafy areas or movements in water. Sign that may be smelled includes the most notable scents of animal urine and skunk. Obviously the ability to see, hear or smell other sign is dependent on the senses of the collector.

Sample Collection:

Upon finding a scat sample a field data sheet is completed. Site name, site description, site location, watershed, date, time, collector and air temperature are all recorded. Site name is in the following format: X#YYMMDD. X is either a two or three letter code for the animal/bird. The following list gives the codes are used for animals and birds under study.

Animal Name	Code
Mouse (White-footed and Deer)	MC
Rat	RT
Mole	MO
Vole	VO
Shrew	SH
Bat	BA
Chipmunk (Eastern)	CP
Squirrel (Red and Gray)	SQ
Woodchuck	WO
Porcupine	PO
Muskrat	MU
Beaver	BV
Rabbit (Eastern Cottontail and Snowshoe Hare)	RA
Rabbit (Domestic)	RD
Weasel (Long-tailed, ermine, and Least)	WE
Mink	MI
Marten	MA
Fisher	FI
River Otter	OT
Skunk (Striped)	SK
Opossum	OP
Raccoon	RC
Red Fox	RF
Gray Fox	GF
Coyote	CO
Dog (Domestic)	DO
Cat (Domestic)	CA

Animal Name	Code
Bear (Black)	BE
Deer (White-tailed)	DE
Moose	MS
Dairy Cow	CA
Septic System (Human)	SS
Horse	HO
Bird Name	Code
Canada Goose	GE
Wild Turkey	WT
Cormorant	CM
Gulls	GL
Snow Goose	SG
American Robin	AR
Crow (American and Fish)	CR
Swan (Trumpeter)	SW
Woodcock	WK
European Starling	ES
Grouse	GR
Gulls	GL
Chicken	CH
Duck	DU
Pigeon	PN
Starling	ST
Tern	TN
Unknown Animal/Bird	UNK

The scat of "unknown" origin can often be grouped as either animal or bird. On bird scat or droppings there is often a nitrogenous "white-wash" coating.

The following information is also recorded on the field data sheet:

A sketch of the site location with major landmarks such as roads, waterbodies, buildings, woods and fields are labeled. This way one can return to the same location if needed.

A habitat description of the surrounding area, especially if follow-up research is necessary to confirm the scat's origin.

A sketch and description of the scat is helpful, especially if the collector is uncertain of the scat's origin. For example scat size, both width and diameter, color, consistency, smell and approximate age of the scat are recorded.

Age of the scat can be determined by several factors. First, weather events such as snowfall and heavy rain storms can often be used to approximate the age of the scat. For example if scat were found on freshly fallen snow which ended three hours earlier you could deduce that the scat was less than three hours old. This would be marked on the data sheet as such. As scat ages, it becomes a less distinctive mass, decreases in moisture content and is covered by debris. Typically, the scat age can be broken down into five categories: >72 hrs (old), <72 hrs, <48 hrs, <24 hrs, and fresh, often accompanied by a positive identification of the animal as it deposited the scat.

The scat is gathered using a "whirl-pak" bag to minimize possible contamination of the sample. If the scat was deposited on a leaf, bark, small stone etc., it is often helpful to pick up the debris with the scat on it and drop the scat in the "whirl-pak" bag. The "whirl-pak" is sealed and labeled with the site name (X#YYMMDD) and the initials of the sample collector.

Sample Identification:

When scat is located sign is a critical part in identifying the responsible animal/bird. If the animal or bird is no longer in the area, sign which can be seen such as tracks, runs, feed remains/areas, caches, rubs, hair, fur, feathers, lays, nests and dens or smelled such as animal urine are most helpful. Animal or bird tracks when present are often the best type of sign that can be used. A sketch of the track along with track pattern can be compared to known track patterns described in reference guides. Two reference guides used to assist in track and scat identification are:

Rezendes, Paul. Tracking and the Art of Seeing: How to Read Animal Tracks and Sign. New York: Harper Collins Publishers, Inc. 2nd Edition, 1999.

Murie, J. Olaus. A field Guide to Animal Tracks. The Peterson Field Guide Series. Boston: Houghton Mifflin, 1954, revised 1974.

Sample Preservation and Transport:

After scat has been collected it is stored on ice or ice packs in a cooler no more than 48 hours until lab delivery. All samples are analyzed at the UNH Jackson Estuarine Laboratory. Upon delivery of the samples, the date and time is also recorded on the field data sheet.

Appendix C Standard Procedure for Detection of Total Coliforms, Fecal coliforms, *Escherichia coli* and Enterococci from Environmental Samples

September, 2002

Latest Revision

May, 2003

Prepared by:
Stephen H. Jones & Tamara Bryant
Jackson Estuarine Laboratory
University of New Hampshire
Durham, NH 03824

_____	_____
Stephen Jones Date	**Tamara Bryant Date**
Author, Lab Director and QA Officer	**Co-Author**

Table of Contents

INTRODUCTION

Various bacterial species and groups of bacteria have been used as indicators of fecal contamination in surface water, groundwater and food. In New Hampshire, state laws dictate the use of 4 different bacterial indicators for use for classifying different types of water. Total coliforms are used for groundwater and some waste water treatment facility (WWTF) permitted discharges, fecal coliforms are used by the NH Shellfish Program for classifying shellfish harvesting areas, enterococci are used for classifying recreational marine and estuarine waters and *Escherichia coli* is used for freshwater recreational waters. The microbiology lab at the Jackson Estuarine Laboratory has conducted projects and has otherwise worked closely with various state agencies concerned with surface water quality in the Seacoast region of NH. Protocols have been used and modified over the past 15 years for the detection and enumeration of different bacterial indicators of fecal contamination. The most recent protocols are presented in the following sections.

This Standard Operating Procedure also includes descriptions of sampling and media preparation. The basic approach is to collect water samples in sterile containers from the field and transport them on ice to the lab as soon as possible. The water samples are filtered through membrane filters and the organisms caught on the filters are grown to colonies on indicator specific media and conditions. The colonies showing the indicator-specific reaction on the agar media are enumerated following appropriate incubation times.

I. Space Requirements

1.1 Specimen Collection.

Not applicable.

1.2 Specimen Intake, Processing and Detection.

This area should include 2 meters of counter space with shelves for storage, and equipped with a water source and a refrigerator. A small area must be designated “clean” for paper work for the prevention of contamination to yourself and others.

1.3 Biochemical Preparation.

This area should include approximately 3.5 meters of counter space with shelves, a readily available de-ionized water supply, an autoclave, storage for biohazard waste, and a large sink.

II. Equipment Requirements

2.1 Specimen Collection.

Laboratory van and/or boat for access to sites, devices for reaching and sampling from surface water.

2.2 Specimen Intake, Processing and Detection.

Autoclave, balance, vacuum pump, filter towers, vortex, Stomacher, computer system for database management, printer, 44.5•C incubator, 35•C incubator, 41•C incubator, thermometers, 4•C refrigerator, ice chest, alcohol burners, loops, scissors, forceps, pipette pump.

2.3 Biochemical Preparation.

Autoclave, test tube washer, hot plate stirrers, alcohol burners, 4•C refrigerator, -22•C freezer, Parafilm®, balance, vacuum pump, filter towers, filter membranes, vortex, pH meter.

III. Chemicals and Supply Requirements

3.1 Specimen Collection.

1000 ml sterile Whirlpac® bags, or autoclavable plastic bottles, waterproof gloves, sterile gloves, permanent marker, cooler and ice, datasheets.

3.2 Specimen Intake, Processing and Detection.

filter membranes, cellulose pads, Buffered peptone water, de-ionized water (DI) DEPC treated DI, goggles, sterile gloves, pipettes of various volumes, graduated cylinders, sterile cellulose pads, Petri dishes containing agar media,

3.3 Biochemical Preparation.

Autoclavable flasks (25 ml- 4000 ml), beakers (10 ml- 500 ml), test tube racks, 50 ml test tube with caps, 13 ml test tubes with caps, stir bars, 15 mm Petri dishes, 3 mm Petri dishes, weigh boats, 0-10 µl pipette,10-100 µl pipette, 100-1000 µl pipette, 1 ml-10 ml pipette, pipette tips for each size pipette, autoclave tape, aluminum foil, indole, mTEC, Mac Conkey, Oxidase, Tryptic Soy agar, Tryptic Soy Broth, Simmon's Citrate, Urea Agar, Urease, Methyl Red, Voges-Proskaur, DEPC treated de-ionized water.

IV. Biochemical Media, Solutions, Preparation and Storage

4.1 Media

All media is to be prepared in a sterile fashion under a hood, lightly covered with tin foil or foam stoppers, wearing gloves, lab coat, autoclave mitts, goggles and tie backs for those with long hair. Store the media agar side up to prevent condensation and at 4•C in plastic sleeves (Atlas and Parks, 1993).

4.1.1 Mac Conkey Agar (Mac)

50 g of Mac Conkey
1000 ml DEPC DI
Mix and boil to dissolve
Autoclave
Dispense to small plates flaming the lip of the flask between plates.

4.1.2 mTec Agar

45.3 g mTec agar
1000 ml DI

Mix and boil to dissolve
Autoclave
Dispense to small plates flaming the lip of flask between plates.

4.1.3 Simmon's Citrate (SimCit)

24.2 g of Simmons Citrate
1000 ml DEPC DI
Mix and boil to dissolve
Autoclave
Dispense to small plates flaming the lip of the flask between plates.

4.1.4 Tryptic Soy Agar (TSA)

40 g Granulated TSA Agar
1000 ml DI
Mix and boil to dissolve
Autoclave
Pour to large plates flaming the lip of the flask between plates.

4.1.5 Urea Agar

29 g Urea Agar Base (in 5ºC)
100 ml DEPC DI
Filter sterilize/DO NOT HEAT
*In **separate flask** suspend:*
15 g Granulated Agar
900 ml DI
Autoclave/Cool to 55ºC
Add Filtered Urea Agar Base
Mix well and pour into small plates flaming the lip of the flask between plates.

4.2 Solutions

All solutions are to be prepared in a sterile fashion under the a hood, wearing gloves, lab coat, goggles, autoclave mitts and tie backs for those with long hair (Atlas and Parks, 1993).

4.2.1 Buffered Peptone Water (BPW)

2.8 g Na2HPO4 (Sodium Phosphate Dibasic)
1.2 g KH2PO4 (Potassium phosphate Monobasic)
4.0 g NaCl
8.0 g Bacto peptone
800 ml DEPC DI
Adjust pH to 7.2 with HCl
Dispense 9.6 ml into large tubes and cap
Autoclave
Store at 4•C

4.2.2 Brain Heart Infusion Broth

37 g Dehydrated Brain Heart Infusion Powder
1000 ml DI
Adjust pH to 7.4±.02
Dispense 10 ml into 20 ml tubes
Cap and Autoclave
Remove and cool to room temperature then store at 4ºC

4.2.3 EC MUG

29.68 g Dehydrated EC medium with MUG
800 ml DI
Adjust pH to 6.9± .2
Carefully dispense 10 ml in to 20 ml tubes containing inverted Durham tubes
Remove and cool to room temperature then store at 4ºC

4.2.4 Indole Reagent

75 ml Iso-Amyl Alcohol
25 ml conc. HCl
pH to <6.0 then add:
5 g p-dimethylaminobenzadehyde
Store at 4ºC

4.2.5 LT Broth

28.48 g Dehydrated lauryl tryptose broth
800 ml DI
Warm to dissolve
Adjust pH to 6.8 ± .0
Dispense 10 ml into 20 ml tubes containing inverted Durham tubes
Autoclave
Store at 4ºC

4.2.6 MRVP Broth (Methyl-Red, Voges-Proskauer)

5.0 g Glucose
5.0 g K2HPO4
3.5 g Pancreatic digest of casein
3.5 g Peptic digest of animal tissue
Add all components to 900 ml of DI.
Mix to dissolve
Bring to 1000 ml
pH to 6.9 at 25ºC
Distribute 10 mls into 50 ml tubes and cap
Autoclave
Store at 4ºC

4.2.7 MRVP Indicator Solution

0.1 g Methyl red
300 ml 95 % Ethyl alcohol
Bring to 500 ml with DI
Filter sterilize
Store at 4ºC

4.2.8 Oxidase Reagent 1%

1 g Tetramethyl-p-phenylenediamine dihydrochloride
100 ml DI
Filter sterilize
Store in dark area at 4ºC

4.2.9 Tryptic Broth for Indole

80 g Tryptic Soy Broth
1000 ml DI
Warm to dissolve
Dispense 5 mls to small tubes and cap
Autoclave
Store at 4ºC

4.2.10 Urea substrate (for use with mTEC)

4 g Urea pellets
200 ml DI
0.02 g Phenol Red Indicator
Mix to dissolve
Adjust pH to 5.0 with dilute HCl (10%)
Filter sterilize
DO NOT AUTOCLAVE
Store at 4ºC

4.2.11 Voges-Proskauer Indicators

Difco VP-A # 261192
Difco VP-B # 261193
Use per manufacturers instructions

4.2.12 Cryoprotectant

Solution 1:
8.5 g NaCl
0.65 g potassium phosphate dibasic
0.35 g potassium phosphate monobasic
1000 ml DI
Autoclave and cool to room temperature
Solution 2:
50 ml autoclaved glycerol, cooled to room temperature
50 ml DMSO
Aseptically mix 800 ml of Solution 1 to all of Solution 2
Store at 4ºC

Hints

When boiling any agar media it is wise to keep an eye on the foam that forms on the surface of the media. As the temperature increases in the flask the foam rises (Atlas and Parks, 1993). When the foam is one inch thick quickly remove the flask from the stir plate. This will prevent the media from boiling over. Put the media in the autoclave as soon as possible to prevent premature setting.

V. Specimen Collection

5.1 Water Samples

With a gloved hand, submerge 100 ml Whirlpac® bag 10-30 cm below the water surface in a direction facing the current and open. For plastic bottles, submerge the bottle with gloved hand in a direction facing the current and

remove cap. In a boat, sample from the upstream side. Care must be taken to avoid disturbance of the surrounding waters prior to or during the sample retrieval. Fill the bag or bottle to capacity and twist the bag closed or re-cap the bottle before surfacing**.** The data sheet should be made of Write-In-the Rain® paper, filled out completely with a Write-In-the Rain® marker and those spaces not applicable crossed off. Record the time**,** date**,** conditions**,** and collector's initials. Put sample on ice and transport.

5.2 Finding and Identifying Scat

There are general approaches to locating scat, and the details of the method used are presented in the NHDES SOP for identification and collection of scat samples (Appendix 2). Knowing the type of habitat that a certain animal resides is critical. A large broad sweep of a field and the surrounding transitional zone is an excellent place to start. Riparian zones often provide a wide variety of scat. Try to identify paths to water and food sources. Temporal bodies of water offer seasonal scat collection. One must also remember that some animals mark territory by defecating or urinating on conspecific scat. A witnessed event is the best identification, but in the wild very rare. Identification of scat can be assisted with the aid of guide books.

5.3 Fecal Samples

Fecal samples should be collected fresh, this reduces the chance of contamination, resource competition, and transformation. Samples that are very dry, found after a rain event, or that show signs of deterioration should not be collected.
Invert Whirlpac® bag over gloved hand and pick up quantity of ***fresh*** fecal specimen. Make sure the sample is as debris free as possible. Revert bag over hand and feces, twist shut. The data sheet should be made of Write-In-the Rain® paper, filled out completely with a permanent marker and those spaces not applicable crossed off. Record date, time, sex (if possible), location, species/breed (using a species code list, appendix 2) and the collectors' initials. Put sample on ice for transport and processing.

5.4 Preparation for incoming fecal and water samples.

Prior to receiving the samples the area should be disinfected. The log book with date and time of sample arrival should be ready for entries. Check the samples against the original collection sheets making sure that all samples have the correct information on their respective containers. Record the samples and their conditions into the log book and have the person delivering the specimens sign the book.

VI. Specimen Intake

6.1 Acceptable Samples

Samples of water should be in water tight containers preferably in a secondary sealed plastic bag. Containers should be labeled with time and date of sample collection, site number and sample collector's name. The water samples have to be analyzed within 2 hours of receipt in the lab. If this holding time is exceeded, then any data for analysis of such samples need to be "flagged", or labeled in a way to reflect this violation of sample integrity.

Fecal samples should be fresh in nature with minimal debris attached. If it appears that a sample has been compromised or has compromised others during transport it/they should be discarded. It is important to note that the integrity and homogeneity of the samples should be without question. A customized Laboratory Management System (see 6.2) should be in place to track samples and analytical data. These data may include: Sample number that is unique to that site, date received, sample descriptions, additional comments, notations about special handling, and name of person delivering samples.

6.2 Specimen Sample Log Sheet

A log book of collection sites, dates the site was sampled, the type of specimen collected, and the date and time of receipt of the sample in the lab should be maintained. Two copies for each sample is recommended. A log book of samples received into the lab and the condition of the samples should also be maintained. A spreadsheet database should be utilized for tracking the specimen and its isolates through the laboratory procedures.

Occasionally, sample analysis requires use of chain of custody sheets for some clients. The procedure is to sign the sheets as required and to take a copy for our laboratory records.

6.2.1 Sample Log Sheet

COLLECTION DATA LOG SHEET

Site Name:
Type of Sample
Site Description:
Fecal
Water
Animal Species:
Location:
Water Temp:
% DO Saturation:

DO:
pH:
Conductivity:
Location:
In stream
Seep
Swale
Storm Drain
Other:
Street:
Town:
Watershed:
Date:
Time:

Sampled by:

Parameters
Weather:
Air Temp:

Flow Rate:
Comments and Sketch/Description

Delivered to lab by:
Date:

Time:
Received by:

VII. Detection and Biochemical Confirmation Methods

7.1 Water Samples

Use flame sterilize forceps dipped into alcohol to aseptically place a sterile gridded 0.45 µm membrane filter on the filter base of a sterile 250 ml filter and attach magnetic filter tower. Vigorously shake the sample bottle or bag at least 30 x and measure out volume to be filtered either in a sterile graduated cylinder or by using a sterile pipette. If the sample is turbid or is suspected of having a high colony count, dilutions of a water sample may be necessary. Add one ml sample to 9 ml sterile BPW and decimally dilute from 10^{-1} to 10^{-7}. Pour up to 100 ml of a sample into the filter tower and conduct routine filtration at 25 millibar until all water has passed through the filter. Turn the vacuum pump off and aseptically remove the filter using sterile forceps.

Positive and negative samples are to be run with each sample set. These include positive samples of enterococci, total coliforms, fecal coliform and *E. coli*, and negative samples for total and fecal coliforms and enterococci. If the results of the positive or negative controls indicate either contamination or culture problems, all sample results will be discarded and samples will be reanalyzed, if holding time requirements are not exceeded.

Field duplicates are routinely collected as part of projects. Colony counts of positive field samples, as well as laboratory duplicate analyses, are expected to agree within 5%.

Each quantification procedure for the different bacterial indicators has specific verification procedures that are followed, and the procedures used at JEL are exactly as described in Standard Methods (APHA 1998). Counts are then adjusted based on the percent verification of these results. Membrane filtration methods require monthly verification of the identity of 10 colonies from one positive sample, as well as representative colonies of non-positive colonies. All positive and negative total coliforms, fecal coliforms and *E. coli* colonies are verified by inoculation of LT and EC-MUG broths to check for lactose fermentation at 35°C, lactose fermentation at 45°C and ß-glucuronidase activity. For enterococci verification, the colonies are streaked to BHI agar, growth is transferred to BHI broth. The 24 h suspension is tested for catalase activity using H_2O_2 and checked microscopically for cocci and gram stain. Catalase negative, gram positive cocci cultures are then transferred to bile esculin agar (35°C), BHI broth (44.5°C) and BHI broth + 6.5%NaCl (35°C) to verify cultures as fecal streptococci and enterococci.

7.1.1 Detection of Total Coliforms

Place the filter onto an M-Endo medium plate, grid side up, by rolling the paper onto the agar surface to minimize air bubbles under the filter. Incubate all plates inverted in incubators at 35±0.5 °C for 22-24 hours (APHA, 1998). Count the colonies that are pink to dark-red and have a metallic surface sheen for each sample/site at best (or all) dilutions (10-30 readable colonies) and record as total coliforms.

Pick an isolated colony from a plate from each sample batch and inoculate **Mac Conkey agar**, **Trypticase Broth for indole**, **Urea agar**, **MRVP Broth** and **Simmons Citrate agar,** incubate at 35±0.5 º C overnight. Conduct **Oxidase** test (looking for no color change) on isolates that are **Citrate negative** (no growth, no color change), **Urea negative** (no color change), and **Indole positive** (pink to violet color), **MRVP** (red color change), **Mac Conkey positive** (pink colonies). These are confirmed *E. coli* colonies, the target species for total coliform analyses.

7.1.2 Detection of Fecal Coliforms and *E. coli*

Place the filter onto an **mTEC** medium plate, grid side up, by rolling the paper onto the agar surface to minimize air bubbles under the filter. Incubate all plates inverted in incubators at 35±0.5 °C for 2 h and at 44.5±0.2 °C for 22 hours (USEPA, 1986).

Count the yellow colonies for each sample/site at best (or all) dilutions (10-30 readable colonies) and record as fecal coliforms (Rippey et al., 1986). Remove top of Petri dish and invert onto counter. Place cellulose pad in lid and pipette 2.0 ml of **Urea substrate solution** onto pad. Roll filter onto pad to discourage air bubbles, cover and incubate for 10-20 minutes at room temperature. Count the yellow/yellow brown colonies using a magnifying lens and record as *E. coli*.

Pick an isolated colony from a plate from each sample batch and inoculate **Mac Conkey agar**, **Trypticase Broth for indole**, **Urea agar**, **MRVP Broth** and **Simmons Citrate agar,** incubate at 35±0.5 º C overnight. Conduct **Oxidase** test (looking for no color change) on isolates that are **Citrate negative** (no growth, no color change), **Urea negative** (no color change), and **Indole positive** (pink to violet color), **MRVP** (red color change), **Mac Conkey positive** (pink colonies). These are confirmed *E. coli* colonies.

For ribotyping projects, pick up to ten isolated, removable, presumptive *E. coli* colonies (yellow colonies after Urease test) per plate and four quadrant streak to **Tryptic Soy Agar**. Incubate at 35±0.5 °C for 24 hours. Repeat the biochemical tests for confirmation of *E. coli* colonies. Those that meet the above criteria can be re-streaked to **TSA** and incubated at room temperature overnight. Keep presumptive *E. coli* isolates frozen in a **Saline Phosphate Buffer and Cryoprotectant** media at -80°C.

7.1.3 Detection of Enterococci

Place the filter onto an **mE** medium plate, grid side up, by rolling the paper onto the agar surface to minimize air bubbles under the filter. Incubate all plates inverted in an incubator at 41±0.5 °C for 48 h (USEPA, 1986). Transfer membrane filter to the surface of an EIA agar plate and incubate at 41±0.5 °C for 20 min. Count pink-red colonies that form a black to reddish-brown precipitate in the agar below the colony using a magnifying lens and record as enterococci.

Pick an isolated colony from a plate from each sample batch and inoculate **Brain Heart Infusion (BHI) agar**, incubate at 35±0.5 º C overnight. Conduct a catalase and a gram stain test on an isolated colony. For catalase negative/gram positive cultures, transfer a colony to BHI broth and incubate for 24 h at 35±0.5 °C. Inoculate BHI broth (incubate at 45±0.5 °C for 48 h), BHI broth with 6.5% NaCl (incubate at 35±0.5 °C for 48 h) and streak a plate of bile esculin agar incubate at 35±0.5 °C for 48 h). Growth on both media indicates that the colony belonged to the enterococcus group of the fecal streptococci.

7.2 Detection of *E. coli* in Fecal Samples

For fecal samples, add 1 g of feces to 9 ml of **BPW** in a sterile Whirlpac® and place in stomacher on medium for 30 sec. Using 2.5 mls of digest, serial dilute in **BPW** to 10-7. Make sure that each tube is labeled as to the dilution, this reduces error.

Filter 10 mls of all dilutions (except first) of every sample and place on **mTEC agar** that has been labeled with the appropriate dilution. Incubate at 44.5°C for 24 hours.

Count and record yellow colonies for each sample/site at best dilutions (10-30 readable colonies).

Remove top of Petri dish and invert onto counter. Place cellulose pad in lid and pipette 2.0 ml of **Urea substrate solution** onto pad. Place filter colony side up onto pad, cover and incubate for 10 minutes at room temperature. Count and record yellow colonies.

Pick up to ten isolated, removable, presumptive *E. coli* colonies (yellow colonies after Urease test) per plate and 4 quadrant streak each onto separate **Tryptic Soy Agar** plates.
Incubate at 35-37°C for 24 hours.

Pick one isolated colony from each plate and inoculate **Mac Conkey agar, Trypticase Broth for Indole**, **Urea agar, MRVP Broth** and **Simmons Citrate agar**, incubate at 35-37º C overnight.

Conduct **Oxidase** test (looking for no color change) on isolates that are **Citrate negative** (no growth, no color change), **Urea negative** (no color change), and **Indole positive** (violet color), **MRVP positive**, **Mac Conkey positive** (pink colonies). Those that meet the above criteria can be re-streaked to **TSA** and incubated at room temperature overnight.

Keep presumptive *E. coli* isolates frozen in a **Saline Phosphate Buffer and Cryoprotectant** media at -80°C.

7.3 Storage of all bacteria

From **TSA plate** pick one colony and place in to vial. Add 1.0 ml of **buffer/ protectant** mixture. Vortex until colony is dispersed completely in buffer. Label cap with specimen number and original collection date. Record the tray and shelf number into the log book then enter it to the database. Place in labeled cryo-rack and put in -80º C freezer.

VIII. Notes on Quality Control

The JEL Microbiology Laboratory QA Plan provides details of QA procedures required to detection of bacterial indicators. The notes below are additional details specific to these procedures.

8.1 General Laboratory Practices

The first concern of any lab is the safety of its personnel. Each person working in the laboratory is trained in lab safety and will be well informed of any hazardous material they might encounter. A chemical roster is stored in the laboratory and Material Safety Data Sheet (MSDS) folders are stored in the JEL Lab Technician's office and kept up to date. Gloves, goggles, gowns or lab coats are advised. No open toed shoes or shorts are allowed. Personnel that have long hair need to tie it back to prevent injury. All instrumentation, cold units, pipettes, incubators, etc. are routinely calibrated by a qualified instrumentation technician.

8.2 Specimen Collection

All collection devises and receptacles must be sterile. Gloves should be rinsed (water) or changed (feces samples) between each sample collected. If a spatula or other collection devise is used it must be sterile. Feces may be double bagged to insure no contact. Water sample lids should be tightened and each bag/ bottle stored and transported upright. Leaking specimens and others in the same transport container may be cross contaminated and should not be accepted. Care should be taken that no specimen comes in direct contact with any other. If at any time a question of contamination arises, discard the sample.

8.3 Specimen Intake and Processing

The laboratory bench surfaces and instruments are to be decontaminated and or autoclaved prior to introduction of specimens. A daily log of instrument cleaning, and temperature control should be checked off, initialed and displayed in a prominent place. If a specimen has been spilled use the lab approved spill kit and all precautions to prevent contamination. Change pipette tips, forceps, and filter towers after each specimen serial dilution.

8.4 Biochemical Preparation and Detection

Biochemicals are the foundation of accurate indicator identification. If the methods or materials are compromised the results would be in question. Gloves and goggles need to be worn for safety and the reduction of contamination. Those that have long hair should tie it away from the face. Compounds, chemicals and other disposables that are received at the lab should have the receive date and the date opened recorded on the receptacle. It is recommended that media and solutions be made in autoclaved containers, under the hood and autoclaved unless otherwise stated. All disposables should be aliquoted to the appropriate containers. Storage of the disposables described in the media section should be strictly followed. The date and the initials of the person that made the disposable should be clearly written on the container. A weekly check of the plated media and a day-of-analysis aseptic check of the pH of solutions is required. As always use the oldest acceptable media first. Tubes and other glass and plastic ware (pipette tips, graduated cylinders) should be capped, autoclaved and stored in the autoclave bags.

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Appendix D Standard Procedure for Ribotyping *Escherichia coli* from Environmental Samples Using a RiboPrinter®

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Latest Revision
March 11, 2004

Prepared by:
Stephen H. Jones & Tamara Bryant

Jackson Estuarine Laboratory
University of New Hampshire
Durham, NH 03824

Stephen Jones Date	**Tamara Bryant Date**
Author, Lab Director and QA Officer	Co-Author

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PROCEDURE OVERVIEW

Water, wastewater and feces samples are processed for isolating *Escherichia. coli* colonies. Fecal samples are mixed using a stomacher, and all samples are decimally diluted to 10^{-7}. Aliquots (2.5 ml) from each dilution tube are filtered through membrane filters (0.45 µ pore size) and the filters placed onto mTEC agar. The agar plates are incubated at 37°C for 2 h then 44.5°C for 22 h. Following urease testing on urea substrate, each plate is inspected and the plates giving countable (20-60) colonies are used for selecting individual *E. coli* strains for analysis. The *E. coli* colonies chosen are subject to a battery of biochemical tests to confirm their identity as *E. coli*. The procedures used for isolating and identifying *E. coli* strains are according to standard lab protocols (Jones, 2002c; Jones and Bryant, 2002).
A RiboPrinter® is used to process *E. coli* cultures for ribotype determinations. After preparation of the samples, the automated process involves lysing cells and cutting the released DNA into fragments via the restriction enzyme EcoR1. These fragments are separated by size through gel electrophoresis and then transferred to a membrane, where they are hybridized with a DNA probe and mixed with a chemiluminescent agent. The DNA probe targets 5S, 16S and 23S ribosomal RNA genes. A digitizing CCD camera captures the light emission as image data, from which the system extracts a RiboPrint® pattern. This pattern could be compared to others in the RiboPrinter® database for characterization and identification based on densiometry data, although our approach has conformed to other ribotyping studies in using banding patterns as the basis for comparing information from different *E. coli* strains.

Isolate Preparation and Storage

Confirmed *E. coli* isolates are processed for determining ribopatterns. *E. coli* isolates are stored in cryovials at -80°C and re-cultured onto trypticase soy agar (TSA). Cultures on TSA are incubated overnight at room temperature (20°C). Some of the resulting culture is transferred to duplicate cryovials containing fresh glycerol/DMSO cryo-protectant media for long-term storage at -80°C.
Isolates are prepared in accordance with the RiboPrinter® manual instructions (RiboPrinter® Sample Prep Ready Reference). The manual suggests preparing isolates after 18-30 h growth at 37°C; at UNH/JEL, all isolates are cultured on tryptic soy agar and incubated for 18 h at 37°C. Sample batches are not prepared greater than 7 days prior to intended use. Prepared batches to be used within 3 days are wrapped in parafilm, closed in an airtight container and stored at 4°C until used. Sample batches to be used within 7 days of preparation are wrapped in parafilm, stored in an airtight container and frozen at -20°C until use. Storage of sample batches prepared in advance is according to the manufacturer's recommended method.

RiboPrinter® Use

2.1 Entering Sample Batches into the RiboPrinter®

Sample batches (a group of eight isolates) are created according to the RiboPrinter® Ready Reference Manual (p. 17) and appropriate data for each isolate (isolate name, date, watershed, sample type, lot numbers for disposables used, operator initials, and project name) were entered into the RiboPrinter® computer software. Sample batches cannot be submitted to the instrument until all data are correctly entered.

When prompted, all disposables and sample carrier are loaded according to the RiboPrinter® Ready Reference Manual (p. 40). It is recommended that disposable buffer base packs should be incubated at 37°C for at least 24 h before using to prevent air bubbles from forming. At UNH/JEL base packs are incubated for 72 h prior to use (Microbial Characterization System, Qualicon). Sample batches will automatically start once all items are correctly loaded and bay doors are closed. Improper placement or orientation of disposables or sample carrier will stop the batch, and the computer screen will prompt the operator to correct the error before beginning.

2.2 Verifying Completed Batches

Before transfer into GelComparII© (Applied Maths) software, all sample batch images are inspected by the operator and at least one other technician for integrity. Batch images are brought up on the RiboPrinter® computer screen (Microbial Characterization System, Qualicon) and all lanes are inspected for alignment, presence of marker, enzyme activity, and successful normalization of the image. Any problems in either the raw or normalized image are referred to the RiboPrinter® online technical support staff (DuPont-Qualicon), as recommended by the manufacturer. The analytical results are recorded as the digital images of the light emission on the membrane that includes the eight samples in a batch and the four standards. The RiboPrinter® normalizes the results to the standard marker lanes.
The similarity threshold for classification of isolates by the RiboPrinter® is 85% similarity to DuPont bacterial strains; isolates with similarity to *E. coli* of 80-84% are subject to verification as *E. coli* via analysis with API 20 E 24-hour identification system (BioMerieux). Isolates with <80% similarity to *E. coli* and isolates that are not verified as *E. coli* using the API identification system are not included for analysis by GelComparII© software. The normalized image is then ready for transfer to GelComparII©.

Ribopattern Determination

Band Identification

The normalized membrane images of the isolate banding patterns are saved in TIFF format to a 3.5 floppy disk from a dedicated Avitron computer for the Riboprinter® housing Q-nix® (Dupont-Qualicon) software then downloaded to a Dell computer housing GelCompar II© analytical software (Applied-Maths) for analysis. The lanes containing the standards are the first images to be processed, labeled and entered into the memory for optimization of membrane pattern. This is done automatically by the interface subset routine script of Q-nix and GelCompar II©, written by Dupont-Qualicon. The standard marker includes bands of 48 kb, 9.6 kb, 6.3 kb, 3.2 kb, 2.2 kb and 0.99 kb (Dupont-Qualicon Operations User's Guide, p. 5-3).
The densiometry data are processed for band identification. The membrane is viewed for integrity including inspections for any anomalies in gray scale or inconsistencies in the lanes for each isolate (Batch/membrane Failures Q/C).

Assigning Bands

The bands for each isolate are automatically assigned for each lane and the data are saved. Occasionally a band for an isolate must be manually assigned when the GelComparII© and RiboPrinter® interface fails to recognize a band. See the GelComparII© manual for further details and instructions (p. 43).

IV. Source Species (Fecal Sample) Library Construction

Each project is assigned an exclusive library. Following manual instructions, the names of the library and its sub-categories are entered into GelcCompar II©. The fecal isolates are grouped in the library sub-categories alphabetically by species then by watershed within a general area (NH, Maine, Region) as instructed in the GelCompar II© manual (p. 112). Once all the isolates of a project have been entered into the library they are analyzed to identify clones using Dice's coefficient, cluster analysis by the un-weighted pair group method by arithmetic averaging (UPGMA), Optimization of 1.5 and Tolerance of 1.0. Only single copies of cloned patterns are maintained in the library so that only unique patterns are included.

V. Identification of Source Species for Water Sample Isolates

Analyzing an Isolate Ribopattern

The banding pattern data for a water sample are selected from a project database containing ribopattern data in units of membrane/sample batches and compared to an appropriate host library. All analyses conducted for identifying

source species are based on the GelComparII Comparative Analysis of Electrophoresis Patterns, Version 2.0, Manual (Applied Maths, Kortrijk, Belgium). A host library and an isolate are selected according to the instructions in the GelComparII manual, quality factors are calculated and the process continued according to the manual instructions. Comparisons are done as described in the manual, using Dice's coefficient. A UPGMA dendrogram is chosen to display the relationship of the isolate to its closest match and others in the library, using settings of 1.0% band tolerance and 1.5% optimization. Both of these parameters are used to adjust the ability to differentiate between bands for the degree of accuracy desired, and also to compensate for possible misalignment of homologous bands caused by technical problems. This allows for banding patterns for water samples to be compared against a library of banding patterns for isolates from known source species to find the best match and thus identify the source species.

The calculated similarity index for the unknown and the most closely matching source species pattern is also subject to interpretation, depending on the conditions of the study and the data available. The source species profile with the best similarity coefficient at a given set of optimization and tolerance settings is accepted as an indication of the possible source species for the water sample isolate. The identification of the source species is considered successful if the value calculated for a given water isolate is equal to or greater than the predetermined threshold value; if the calculated value is below the threshold similarity index, the water sample isolate is considered to be of unknown origin. Further interpretation is conducted based on matching of patterns at similarity indices equal to or greater that the threshold level.

The ultimate decision on what similarity index level should be used is based on a number of criteria. First, we consider the inter-gel variability by using Dice's coincidence index to analyze patterns for our *E. coli* positive control. Other factors, including the size of the database and the data quality objectives for each project are also considered.

5.2 Data Analysis

All data are analyzed with GelComparII© software on a Dell computer, where the source species databases are also stored. Hard copies of ribotype patterns and similarity coefficients for a given water sample isolate and the most closely related source species are printed for interpretation. Interpretation is done by visual inspection of hard copies and accompanying graphical representations of the data are done in MS Excel on Macintosh G-4 computers. All data are stored in hard copy and in disk format as per Dupont-Qualicon Operations User's Guide (p. 6-26).

VI. QA/QC

Membrane Transfer Failure

If image data for a membrane fail to transfer (for) gray scale or if there are lane anomalies, the data are completely deleted from the GelComparII© database and the computer. The 3.5" floppy disk is erased and re-formatted, the membrane data are re-transferred from the RiboPrinter onto the "clean" 3.5" disk and data are re0entered into GelComparII©. This has always remedied the problems.

Isolate Failures*

For every isolate to be considered *E. coli* it must match Dupont -Qualicon Identified (DUP-ID) strain *E. coli* at 80% similarity or greater. If an isolate fails to meet this standard or if the DUP-ID is not *E. coli* an API 20E identification system test is performed according to the manufacturers instructions. If the isolate is found to be *E. coli* it is entered into the database; if it fails the isolate pattern is excluded from the database.

Batch/Membrane Failures*

Upon receiving a message that two or more bands on a marker lane have failed a confirmation with another on-site technician is made. The batch/membrane may have failed, but the data may still be valid. The image and data need to be manually extracted with the assistance of the Dupont-Qualicon technical support staff.

All other membrane failures are to be called in to the Dupont-Qualicon technical support staff. A hard copy of each failure is printed out and documented in a lab book, the technician's name that was contacted, and the outcome.

Daily Systems QC*

Every morning when then instrument is started it self initiates a cleaning and homing program. This runs purified distilled water through the pipettes and lines, homes the motors, pipettes, arms, heaters and focuses the camera lenses.

Monthly QC*

American Type Culture Collection Strains of *Listeria innocua* #51742, *Salmonella ser. infantus* #51741, *E. coli* # 51739, *Staphlycoccus aureus* # 51740 performed every month, as recommended by the manufacturer, and before the start of each new project. Briefly: Each lyophilized culture is re-hydrated in TSB overnight at 37ºC streaked onto a TSA plate and incubated overnight at 37ºC and is prepared per manufacturer's instructions for processing on the RiboPrinter®.

6.6 Clean Instrument QC*

CI/QC performed at the start of every new project and when the instrument has been idle for three days. Briefly: click on maintenance, click on diagnostic batches, click on clean instrument.

6.7 Three Month Maintenance*

Every three months it is recommended that a 10% bleach rinse be performed per manufacturer's instructions. Briefly: Make 4L of a 10% bleach solution, place 450µl of the 10% solution in a clean enzyme prep a pack carrier. Place the remaining bleach solution in the bulk water container and follow the clean instrument QC protocol twice. Once complete remove the bleach solution from the bulk water container unit and rinse with DI water two times. It has also been recommended that ST1 and ST2 be cleaned at the same time and you will need technical support for assistance.

6.8 Preventative Maintenance (PM)*

Twice yearly contractual visit from manufacturer to replace warranted parts, as recommended by manufacturer.

*RiboPrinter®
Microbial Characterization System
Operations User's Guide and
Data Analysis User's Guide
Qualicon

Appendix E Sampling Station Identification Form

Sampling Station Identification Form

Note: Shaded items are ultimately required. Form Completed

Project | Station ID (15 char max) | Alias ID | Station Name

Transect | Town (no village names) | State (circle one): NH ME MA VT Canada | Date Established ___/___/___

Station Type (circle one)

Canal - Drainage	Culvert	Lake	Riverine Impoundment	Wetland - Estuarine, emergent
Canal - Irrigation	Drain Manhole	Land	Seep	Wetland - Estuarine, forested
Canal - Transport	Estuary	Landfill	Spring	Wetland - Estuarine, scrub-shrub
Catch Basin	Facility - Industrial	Land Runoff	Storm Sewer	Wetland - Lacustrine, emergent
Cave	Facility - Municipal Sewage (POTW)	Mine/Mine Discharge	Tidal Swale	Wetland - Palustrine, emergent
CERCLA Superfund	Facility - Municipal Water Supply	Ocean	Waste Pit	Wetland - Palustrine, forested
Channelized Stream	Facility - Other/combined	Pipe	Waste Sewer	Wetland - Palustrine, moss-lichen
Combined Sewer	Facility - Privately owned non-industrial	Reservoir	Well	Wetland - Palustrine, scrub - shrub
Constructed Wetland	Gallery	River/Stream		Wetland - Riverine, emergent

Waterbody Name | Designated River Reach (list on other side)

Related Lake | Final Discharge Location (Use Watershed Assistance) | Tidal Station | Water Depth | Units (Circle one): in/ft/cm/m

Station Description:

Directions to Station:

Date Located ___/___/___

If located by GPS:
Latitude (Ex:DD MM SS.ss) | Longitude | GPS File Name | GPS Unit/Serial # (list on other side) | Corrected? Yes No

Locational comments:

If located by other method:

Method of Location (circle or enter):
Interpolation - Map | Land-Survey
Interpolation - Photo
Interpolation - Satellite
Other: _____

Map Scale (circle or enter)
1:24,000/25,000
1:100,000
Other: _____

Datum (circle or enter)
NAD 1927
NAD 1983
WGS 1984
Other: _____

Elevation Information:
Elevation | Units: ft/m

Method (Circle one)
Map Interpolation Digital (DEMs)
Differential Mode GPS
Absolute Mode GPS
Conventional Survey
Public Land Survey
Altimeter

Datum (circle or enter)
NGVDD 1929
NAVD 1988
WGS 1984
Local Tidal Datum
Mean Sea Level
Other: _____

Site Diagram (or attach map with location marked)

Designated River Segments:

- Ashuelot
- Cold
- Connecticut
- Contoocook/North Branch
- Exeter
- Isinglass
- Lamprey
- Lower Merrimack
- Pemigewassat
- Piscataquog
- Saco
- Souhegan
- Swift
- Upper Merrimack

GPS Units:

Make	Model	Serial#	Section
Garmin	GPS III	40157743	Biomonitoring
Garmin	GPS III Plus	92186038	Watershed Assistance
Garmin	GPS III Plus	92177955	Water Quality
Magellan	320	23857	Shellfish
Trimble	GeoExplorer II	0010004LQ8	Biology
Trimble	GeoExplorer II	0010004LQ2	Biology
Trimble	GeoExplorer II	ZB970	Watershed Assistance
Trimble	ProXL	3450A00313	Data Management

H:\Data Management\Water quality database\station form new.pdf

Appendix F Water Sample Collection Field Sheet

Project Name:
Contact Name and Phone Number:

Samplers: Date:			Current Weather:_____ Air Temp:_____ Start of Last Rain:	
Site ID	Time of Sample	Water Temp (C°)	Water Flow / Level *	Comments

*Water Flow / Level: Very Low, Low, Medium, High, Very High

Sample Chain of Custody: Samples delivered to lab by:_____ Date: _____ Time: _____

Received by: _____ Date: _____ Time: _____
Page _____ of _____

Appendix G Fecal Material Sample Collection Field Sheet

Project Name:
Contact Name and Phone Number:

Samplers:			Date:		
Sample ID	Time of Sample	Approx. distance to surface water	Animal Species & age of scat	Habitat Description	Field Notes and Sketch

Sample Chain of Custody:

Samples delivered to lab by:_____ Date: _____ Time: _____
Received by: _____ Date: _____ Time: _____

Appendix H MST Project Water & Fecal Material Monitoring QA Sheet And Delivery Form

Project Name: _____

(*Use format: (Agency/organization) (Project focus) (Start year). Example Project Name: DES Little Harbor Stormdrains 2003*)

Watershed: Great Bay Little Harbor Atlantic Coast Rye Harbor Hampton/Seabrook Harbor
And Subwatershed/Waterbody (specify):_____

Sampling Day Info.
Samples Collected by:_____ Collection Date:_____
No. Sites Sampled:_____
Matrix (circle one): water fecal material

Air Temp (^{o}C):_____ Weather Conditions: _____

If tidal waters: Portland Low Tide Time_____

Chain of Custody
Transported to _____ _____by_____on_____
(name/org.) (name) (date)

Transported to _____by_____on_____
(name/org.) (name) (date)

Lab QA
Delivery Date:_____ Delivery Time:_____ Delivery Temp._____°C

Samples acceptable? (yes/no)_____ Samples accepted by:_____

If no, state problem:_____

Analysis Date:_____ Analysis Time:_____ Analysis Temp._____°C

Analyst:_____ Report Verifier:_____ Date of Report:_____

Parameter: fecal coliform *E. coli*

Data Report Sent to DES
Sender's Name_____Date_____

DES Data Entry
Data Entered by_____ Date_____
Entry Verified by_____ Date_____
Final QC Check by_____Date_____

Appendix I Quality Assurance Plan: Microbiology Laboratory at the UNH-Jackson Estuarine Laboratory

September, 2002

Latest Revision
June 18, 2003

Dr. Stephen H. Jones
Jackson Estuarine Laboratory
University of New Hampshire
85 Adams Point Rd.
Durham, NH 03824

JEL Microbiology Lab Director:
& QA Officer: _____

Signature / Date
Stephen H. Jones

November 26, 2002 version reviewed and approved by Arthur Clark, EPA, on 12/2/2002.

Microbiology Laboratory Quality Assurance Plan
Jackson Estuarine Laboratory
November, 2002

The Jackson Estuarine Laboratory's Microbiology Laboratory is a research laboratory that supports a variety of different projects on an ongoing basis. The lab also does some analysis for contracts, but this is not the major activity of the lab. As part of these projects, the lab routinely analyzes environmental samples for a variety of different fecal indicator bacteria, including total and fecal coliforms, enterococci and *Escherichia coli*. The procedures for these analyses are described in an SOP recently updated in September, 2002 (see below: Jones and Bryant, 2002). Various types of environmental samples are processed for analysis, including sediments; soils, feces, wastewater and water, but the vast majority of samples processed are surface water. Other bacteria have also been of interest for some past projects, including various pathogenic vibrio species, *Clostridium perfringens*, and a variety of environmentally relevant pure and mixed cultures.

1. Laboratory organization and responsibility

Table 1. Personnel Responsibilities and Qualifications Relative to Microbiology Laboratory

Name and Title	Responsibilities	Immediate Supervisor
Steve Jones, Ph.D. Principal Investigator on all lab projects	Administration and oversight on all projects, personnel training, QA Manager on many projects	NA
Andrew Beach, Laboratory Technician	Collection of water samples for microbial analysis and data compilation on one project	Steve Jones
Tamara Bryant, Research Technician II	QA Development Officer for ribotyping projects; training of student workers	Steve Jones
Danielle Morin, Laboratory Technician	Collection of water samples for microbial analysis and data compilation on one project	Steve Jones
Scott Nolan, Laboratory Technician	Collection of water samples for microbial analysis and data compilation on one project	Steve Jones
Bethany O'Hara, Research Technician II	Ribotyping technician and QA of laboratory equipment.	Steve Jones
Acksone Soumpholphakdy, Laboratory Technician	Collection of water samples for microbial analysis and data compilation on numerous projects. Training new students.	Steve Jones

Based on EPA-NE Worksheet #6.

Dr. Jones is the QA manager for most projects and is responsible for ensuring the production of valid measurements and the routine assessment of measurement systems for precision and accuracy (e.g., internal audits and reviews of the implementation of the QA plan and its requirements). The development of QA procedures for ribotyping is an effort headed by Tamara Bryant, with supervision by Dr. Jones.

All job descriptions and employee qualifications are on file in Dr. Jones' office. All personnel are trained by those identified above for different projects to keep personnel updated on regulations and methodology. Dr. Jones keeps records on all the training that personnel receive outside of the laboratory.

List of SOPs with the dates of the most recent revisions

Stephen H. Jones & Tamara Bryant. Standard Procedure for Detection of Total Coliforms, Fecal coliforms, *Escherichia coli* and Enterococci from Environmental Samples. Revised: September, 2002. (based on: APHA, 1998; US EPA, 1986; 1996).

Stephen H. Jones. 1992. Most probable number method for the enumeration of *Clostridium perfringens* in marine sediments, p. 384-387. In, Standard Operating Procedures and Field Methods Used for Conducting Ecological Risk Assessment Case Studies, Mueller, C. et al. (Eds.). Naval Construction Battalion Center, Davisville, RI and Naval Shipyard, Portsmouth, NH. (based on: US EPA, 1996; Calise, 1991; St. John et al., 1982).

Copies of the SOP are on file in Dr. Jones' office and in the main laboratory. All listed SOPs are all reviewed annually and/or revised as changes are made.

3. Field sampling procedures

Microbiological sampling from the field requires sterile containers, either autoclaved plastic bottles with caps or WhirlPak bags. The plastic bottles can be reused, so cleaning involves re-autoclaving for disinfection, thorough cleaning with soap and hot water then rinsing in tap water and deionized water. Surface sediment samples are collected using sterile scoops to remove surface sediment samples that are transferred to WhirlPak bags.

In general, the time interval between water sample collection and analysis is minimized to optimize the reliability of the analytical results. All samples are temporarily stored on ice in coolers in the field to reduce biological activity and changes in the microflora. Water samples can only be held for a total of 8 hours prior to analysis, or, 6 h maximum for transport to the laboratory and 2 h maximum time between arrival of sample at lab and analysis (APHA, 1998). For some projects where screening of samples is done to see generally what levels of bacteria exist, samples may be held for somewhat longer time intervals. All samples are stored in a refrigerator for at until the next day following the initial analysis to allow for re-analysis if the initial analysis was not acceptable for any reason. Data from reanalyzed sample results are flagged and only used for informational purposes. The only time custody forms are required is for projects other than internal projects, where another collaborative entity may require such forms.

All sample containers are checked just prior to analysis to ensure proper labeling, proper containment and that no cross contamination has occurred.

4. Laboratory sample handling procedures

Bound laboratory notebooks are used for entering sample information into the laboratory records. Information is filled out in ink, dated and the person entering the information includes their name on the page(s). These notebooks are stored in the analytical laboratory and records throughout the holding time of the samples are maintained in them. After each batch of samples has been analyzed, the results are recorded into spreadsheet databases on a computer in a room adjacent to the analytical laboratory.

All unprocessed and processed samples are stored in designated areas within a walk-in cooler located adjacent to the analytical area of the laboratory. The temperature of the walk-in cooler is thermostatically controlled to be 4°C but actually ranges between 3-8°C; a chart recorder maintains a record of actual temperatures. UNH facilities personnel periodically check the cooler and maintain it.

Unprocessed and processed samples are stored separately in the cooler, with unprocessed samples remaining in field coolers on the floor and processed samples stored on shelves. All sampling occurs according to predetermined schedules to ensure that holding times will not be exceeded and that incubations and final analyses will occur according to SOP requirements.

Chain-of-Custody procedures are not normally imposed because samples likely to be the basis for an enforcement action are not analyzed in this laboratory. However, occasional samples are received for analysis from other entities that may require Chain of Custody procedures for their own purposes.

Samples collected by other entities and delivered to the JEL Microbiology lab may be rejected if it is determined that they do not meet shipping, holding time and/or preservation requirements. This is determined by review of the datasheet provided to them by our laboratory to see when samples were collected and how they were shipped. Sample originators are immediately notified either by telling the delivery person or emailing/telephoning and providing them with the reasons for the rejection.

5. Calibration procedures for chemistry

There are no chemical analyses performed by the Microbiology Laboratory.

6. Data reduction, validation, reporting and verification

Data in laboratory notebooks are reviewed to ensure completeness of data entry and accuracy of labeling as soon as final analytical results are made. Within a few days, the raw data in the laboratory notebook are initially subject to calculation of average values from laboratory duplicate and any field duplicate analytical results. Two technicians working together conduct this calculation process. The sample average is recorded directly into the laboratory notebook. Sample averages are entered into spreadsheet databases for each project by two technicians: one reads the values from the lab notebook and relates the values to the other who enters the data into the computer. The project database(s) is organized by bacterial indicator, date and sample site, along with any other pertinent sampling date and site-specific data, measured or observed.

Dr. Jones is responsible for evaluating all data. This process includes assessment of database completeness, transcription errors and compliance with procedures. When possible, the data are also evaluated for consistency with previous correlated databases to determine if data are within expected ranges for sites and time of year. Omissions of data in spreadsheets will trigger a search of raw datasheets for missing data or possibly reanalysis of the questionable sample, if possible. If reanalysis is not possible or if data remain missing, invalid or otherwise affected entries will not be incorporated into the useable data set. When results appear to be abnormal, all appropriate project participants will review the available data and discuss the problem in periodic meetings to attempt to identify potential problems in sampling or analyses.

The reporting of analytical results is project dependent. For internal research projects, the data are fully analyzed by the PI and appropriate project technicians or graduate students, and eventually published in reports provided to the funding agency. For contract analysis results, the data are provided to funding agencies in Excel spreadsheets in formats pre-determined by the agency or project participants.

Each quantification procedure for the different bacterial indicators has specific verification procedures that are followed, and the procedures used at JEL are exactly as described in APHA (1998) . Counts are then adjusted based on the percent verification of these results.

Membrane filtration: In general, membrane filtration method verification procedures all require monthly verification of the identity of 10 colonies from one positive sample, as well as representative colonies of non-positive reactions or morphologies. All positive and negative total coliform, fecal coliform and *E. coli* colonies are verified by inoculation of LT and EC-MUG broths to check for lactose fermentation at 35°C, lactose fermentation at 44.5°C and b-glucuronidase activity. For enterococci verification, the colonies are streaked to BHI agar, growth is transferred to BHI broth. The24 h suspension is tested for catalase activity using H2O2 and checked microscopically for cocci and gram stain. Catalse negative, gram positive cocci cultures are then transferred to bile esculin agar (35°C), BHI broth (44.5°C) and BHI broth + 6.5fiNaCl (35°C) to verify cultures as fecal streptococci and enterococci.

Multiple tube fermentation: In general, all MTF procedures are verified by using 10% of positive samples. TC, FC and Ec tests are verified using brilliant green and EC-MUG broths as described in SM 9221 B.3. *C. perfringens* tests are verified by streaking positive tubes to mCP agar and confirming *C. perfringens* by observing characteristic colonies after 24 h of anaerobic incubation at 44.5°C.

7. Quality control

a. Within Sample Batches

Positive and negative samples are to be run with each sample set. These include positive samples of enterococci, total coliforms, fecal coliform and *E. coli*, either *Enterococcus faecalis* or *E. coli*. Negative sample cultures for the fecal indicator bacteria or other target bacteria (vibrio species, *Clostridium perfringens*, etc.) are selected from a variety of different non-fecal and non-target bacterial species that are maintained in the laboratory. In each sample set, duplicate analyses of a positive sample are run by the analyst. Colony counts are expected to agree within 5%. Monthly positive samples are also run in duplicate by the different analysts, and colony counts between analysts are expected to agree within 10%.

b. Precision

Precision for bacterial indicator measurements is typically determined according to Standard Methods 9020 B-8. (APHA, 1998). The range (R) for duplicate samples is calculated and compared to predetermined precision criteria. The precision criterion is calculated from the range of log-transformed results for 15 duplicate according to the following formula:

3.27 × (mean of log ranges for 15 duplicates) = precision criterion

The precision criterion is updated periodically using the first 15 duplicate samples analyzed in a month by the same analyst. If the range of ensuing pairs of duplicate samples is greater than the precision criterion, then the increase in imprecision will be evaluated to determine if it is acceptable. If not, analytical results obtained since the previous precision check will be evaluated and potentially discarded. The cause of the imprecision will be identified and resolved.

c. Media Preparation and Equipment

Various types of sterility controls are included in the different procedures used to detect and enumerate microorganisms. Sterile water is filtered through membrane filters in filter towers prior to use of the filter tower for sample filtration for the first and last samples of a sample batch. The membrane filter is then incubated on the target test media to see if any bacteria are present. Uninoculated dilution

tubes and agar media are incubated along with inoculated media to check for contamination for each batch of samples. If the results of the positive or negative controls indicate either contamination or culture problems, all sample results will be discarded and samples will be reanalyzed, if holding time requirements are not exceeded.

Other QC procedures for lab supplies generally follow SM 9020 B.4 for pH and inhibitory substances on glassware, laboratory reagent water quality, quality of media and reagents and membrane filter integrity. Procedures for preparing, sterilizing, handling and storing media and other equipment are as described in SM 9020 B.4i.1-5.

8. Schedule of internal audits

Dr. Jones conducts periodic (minimum frequency: annually for projects >1 year in duration) internal audits of all aspects of project QA/QC and personnel performance. The timing of performance audits is project specific, and typically occurs in the very beginning of a project, within one month of project analysis initiation, and later in the project after the technicians have established procedural prowess. Any problems are noted, corrective actions are recommended and follow-up audits are conducted to verify compliance with correct procedures. Written records in the form of checklists with details of problems and follow-up audit results are kept in Dr. Jones' office.

9. Preventive maintenance procedures and schedules

The technicians responsible for project or laboratory QC conduct all maintenance and inspection of equipment based on manufacture requirements and specifications. Every day a piece of equipment is used it receives a general inspection for obvious problems. The most common assessment requiring corrective action is maintenance of correct temperatures for incubators. Results of inspections are recorded on datasheets that include date, time, and inspector initials, and completed sheets are on file in Dr. Jones' office. Much of the other equipment used in the Microbiology Lab is not under the direct control of Dr. Jones and is maintained by regular UNH inspections (Autoclave, walk-in coolers, scales, etc.). Lab technicians always check chart recorders and digital read outs on the autoclave and the coolers with each use to confirm correct settings and conditions. Any probelms are reported to the JEL Lab Manager who contact UNH Maintenance for any necessary repairs beyond his expertise. Scales are checked annually by UNH-hired experts and the date, time, results and inspector's initials are recorded on the scale. In addition, microbiological data are inspected within a few days of sample analysis to allow instrument (or user) malfunctions to be caught quickly, and corrected as needed.

10. Corrective action contingencies and record keeping procedures

Unacceptable lab QC checks triggers immediate review of analytical procedures, sample processing and equipment with the technicians involved. Data results from the time period between the previous acceptable lab QC checks are reviewed to determine if there is evidence for accepting the data, otherwise, it is considered invalid. All project-specific personnel are responsible for participating in corrective actions like re-training or learning modified QC procedures to ensure future acceptability. A database of corrective actions is maintained on a computer in the PI's office. The office is either occupied by the PI or is locked and no one else is admitted in.

REFERENCES

American Public Health Association. (APHA). 1998. Standard Methods for the Examination of Water and Wastewater. 20th Edition. American Public Health Association, Washington, DC.

Calise, B.A. 1991. ERL-N Standard Operating procedure. Most probably number method for enumeration of *Clostridium perfringens* in marine waters. ERL-N SOP 1.03.017, 4 pp.

St. John, W.D., J.R. Matches and M.M. Wekell. 1982. Use of iron milk medium for enumeration of *Clostridium perfringens*. J. Assoc. Anal. Chem. 65: 1129-1133.

U.S. Environmental Protection Agency (USEPA). 1996. ICR Microbial Laboratory Manual. Sections X (*E. coli*) and XI (*C. perfringens*). EPA 600/R-95/178. Environmental Protection Agency, Office of Research and Development, Washington, DC.

U.S. Environmental Protection Agency (USEPA). 1986. Test methods for *Escherichia coli* and enterococci by the membrane filtration procedure. EPA 600/4-85/076. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH.

Appendix J Sampling and Analysis Plan For Microbial Source Tracking Projects

A Sampling and Analysis Plan (SAP) is prepared for each project by the Project Manager, reviewed and approved by the Field QA Officer prior to field work, and a copy retained in the Coastal Restoration Supervisor's Office at Pease. A copy of the approved plan is sent to the DES Quality Assurance Manager. The Project Manager is responsible for communicating the SAP and other QA/QC requirements to other field sampling staff that may be working on the project.

The SAP will reference its parent Generic QAPP. Deviations from and stipulations not addressed in the Generic QAPP are incorporated into the SAP. These will include site information, rationale, project description and timetable, and reporting. Additional information is considered and added on a case-by-case basis. Also, the Project Manager is responsible for locating and producing procedures for any deviations and stipulations, in particular, sampling and testing required for a project that is not described in the Generic QAPP. Details should be provided for the elements listed below.

SAP Information

Project name
Date of SAP
Reference to Generic QAPP for Microbial Source Tracking
Contact information of person preparing the SAP

Site Information

Site map
Sampling location map
Personnel identification and organization

Rationale

Problem definition
Historic Data
Conditions of concern

Project Description and Timetable

Sampling design (sampling location information, representativeness, fecal material targets, sampling and analysis method/SOP requirements[1], # of isolates/sample for ribotyping analysis)
Sampling procedures and requirements
Data analysis and interpretation

Reporting

To whom results and discussion are reported and how

A title and approval page will be submitted to the NHDES Quality Assurance Manager with the SAP. The page will include the project title, date of submittal, name and title of person preparing the SAP, name and title of person who receives the SAP for approval, a list of people involved in the SAP and a space for these people to sign and date upon their review of the SAP.

Note 1: If SOPs other than those currently present in the Generic QAPP for MST are used, a copy of the relevant SOP is to be attached to the SAP for documentation purposes.

Appendix K DES Laboratory Services Unit Standard Operating Procedures for Thermometer Calibration

A. THERMOMETERS

The laboratory's thermometers are calibrated against an NIST traceable thermometer on a yearly basis for glass-liquid thermometers and quarterly for dial thermometers. The NIST thermometers are sent to the manufacturer or a service company for recalibration and re-certification every three years. The calibration records are kept in a bound record book. The working thermometers all have identification tags showing the thermometer name and serial number, date of calibration, and correction, if any.

1. Calibration check is performed as follows:

Calibration System: ERTCO Thermometer Calibration System, Model TCS 200-35
Solvent Bath: 50% Propylene Glycol + 50% tap water
Temperature Range: -30 to 100^{0}C
NIST Traceable thermometers: Serial #1977, -1 to 201 ^{0}C, scale divisions 0.2 ^{0}C
Serial #1157, -1 to 101 ^{0}C, scale divisions 0.1 ^{0}C
Serial #F96-146, -36 to 54 ^{0}C, scale divisions 0.2 ^{0}C

Calibration System Operation and Correction Determination:

a. Turn on power by pressing ON button. Wait for self-test to conclude.
b. Select operating temperature by first pressing SET, then use the number pad to enter the temperature. Digits on the display will move to the left. Enter a zero to advance past the decimal point.
c. When the display shows the desired temperature, again press SET to lock it in. The calibration bath will then heat or cool to reach the set temperature. The FLUID read-out tells you the temperature of the bath. Always use the temperature on the NIST traceable thermometer for calibration.
d. Insert NIST and test thermometers into the entry ports of the calibration system. Make sure the bulbs are properly immersed.
e. After the thermometers have reached stable readings, begin recording comparative readings. Take an initial reading, an intermediate reading after 10 minutes, and a final reading after another 10 minutes. Record all readings in the thermometer calibration bench book. Make corrections to the test thermometers based on their final readings compared to the NIST final reading. Prepare identification tags as described above and place on each thermometer. When no correction is needed, the tag lists "-0-".
f. To turn off calibration system, press the OFF button.

B. BALANCES

Analytical balances are serviced on a yearly basis by a certified balance calibration company. The Class S weights are sent to a service company for recalibration and re-certification every three years.

Balances are checked each day of use by the first analyst to use the balance that day. Three weights are used which bracket the range of use. The weights used have been compared to the NIST weights and found to be comparable. An acceptability table is included in each balance log (see below).

C. BALANCE WEIGHT ACCEPTABILITY:

Instructions: Weigh three weights which bracket the range of use (weight of sample plus container). Record results in the Weight Observed column. Record all decimal places. Place a check if the results are acceptable by comparing results to the acceptable range table below. Results must agree with target weight within ±10% for 0.002 g, ±1% for 0.02 and 0.05 g, and ±0.1% for 0.1 g and larger. If outside these limits, notify the QC officer and use an alternate balance. Always clean up after using the balance.

Acceptable Range Table

Target Weight, g	Tollerance Criteria, g	Agreement Percentage
0.002	0.0018-0.0022	±10%
0.02	0.0198-0.0202	±1%
0.05	0.0495-0.0505	±1%
0.1	0.0999-0.1001	±0.1%
0.5	0.4995-0.5005	±0.1%
1	0.999-1.001	±0.1%
5	4.995-5.005	±0.1%
10	9.99-10.01	±0.1%
20	19.98-20.02	±0.1%
30	29.97-30.03	±0.1%
50	49.95-50.05	±0.1%
100	99.9-100.1	±0.1%
300	299.7-300.3	±0.1%

D. PIPETTES:

This method provides instructions for the gravimetric calibration of fixed and variable volume pipettes. Volumetric pipettes are not included in the scope of this method. Pipettes are calibrated quarterly.

1. Equipment
   a. Analytical balance capable of reading to 0.0001 g, preferably with digital read-out.
   b. Deionized water.
   c. Pipettes to be tested and their accompanying tips.
   d. Assorted glassware and weigh boats for holding water.

2. Balance Calibration:
   a. Check balance calibration by weighing 3 Class S weights over the range of the expected weights. Record in balance logbook and on Pipette Calibration Record Sheet.

3. Determination:
   a. Place a receiving beaker or weigh boat on the balance pan. After the balance achieves a stable weight, press the tare button to zero the display.
   b. Pre-wet the pipette tip and then dispense an aliquot of water into the beaker.
   c. Record the weight of the water on the record sheet. Repeat the taring and weighing with two more aliquots.
   d. Continue in this manner with each successive pipette until all have been tested.
   e. Variable volume pipettes will need to be tested at both the low and high end of the pipette volume range.

4. Data Handling Procedures:
   a. Print out or photocopy the Pipette Inventory before starting. Also print out or photocopy as many pages as you will need of the Pipette Calibration Record Sheet. Number the pages of the Record to show the order of calibration check.

b. Calculate the mean of the three weights for each pipette and enter on the record sheet. We assume 1 mL DI water = 1 g. A 2-3% difference from the stated volume is reasonable unless otherwise specified by the manufacturer.
c. Turn in any non-conforming pipettes to the QC Officer for repair or replacement. Notate on the inventory sheet. The QC Officer also maintains records of repair.

5. Quality Control
   a. Class S weights are sent for re-calibration and re-certification once every three years.
   b. Analytical balances are serviced on a yearly basis by a qualified balance service.
   c. Ultra pure deionized water is produced in the lab by a Millipore Milli-Rx 45 RO / ELIX system capable of producing >15 megohm water.

E. GENERAL CALIBRATION OF EQUIPMENT

Analytical equipment is calibrated according to method and/or manufacturer's recommendation. Please refer to individual SOPs for specific notes on instrument calibration.